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COOPERATIVE AGREEMENT NO. DAMD17-94-V-4009

TITLE: Toxicokinetics of Sulfur Mustard and its DNA-Adducts in the Hairless Guinea Pig - DNA-Adducts as a Measure for Epithelial Damage

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REPORT DATE: December 1998

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 1998	Final (1 Jul 94 - 3o Jun 98)

4. TITLE AND SUBTITLE

Toxicokinetics of Sulfur Mustard and Its DNA-Adducts in the Hairless Guinea Pig – DNA-Adducts as a Measure for Epithelial Damage

5. FUNDING NUMBERS

DAMD17-94-V-4009

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

19. ABSTRACT *(Maximum 200 words)*

The toxicokinetics of sulfur mustard (SM) as well as of its major DNA-adduct were studied in male hairless guinea pigs for the intravenous, respiratory and percutaneous routes. The study comprised measurement of the concentration-time course of SM in blood and measurement of the concentrations of intact SM and its DNA-adduct in various tissues at several time points after administration of, or exposure to SM. SM was analyzed in blood and tissues by gas chromatography with large volume injection and mass-spectrometric or pulsed-flame photometric detection. DNA-adducts were measured via an immunoslotblot assay or immunofluorescence microscopy. The intravenous toxicokinetics of SM are characterized by a very rapid distribution phase and a very slow elimination phase. SM partitions strongly to various organs. The toxicokinetics are non-linear with dose. The respiratory toxicity of SM appears to be of a local, rather than a systemic nature, since concentrations of SM in blood could only be found during and after nose-only exposure to 3 LCt50 (2,400 mg.min.m^{-3}). SM could also be measured in blood during and after percutaneous exposure to 1 LCt50 (10,000 mg.min.m^{-3}, estimated). Pretreatment of hairless guinea pigs with the potential scavengers N-acetyl cysteine or cysteine isopropyl ester did not significantly increase the LCt50-value for nose-only exposure to SM vapor. Topical skin protectants 1511 and 2701 protected very well against skin damage resulting from exposure to SM vapor or liquid.

14. SUBJECT TERMS sulfur mustard, DNA-adducts, toxicokinetics, hairless guinea pig, intravenous, inhalation, percutaneous, blood, tissues, bioanalysis, gas chromatography, immunoslotblot, immunofluorescence microscopy, scavengers, topical skin protectants

15. NUMBER OF PAGES 137

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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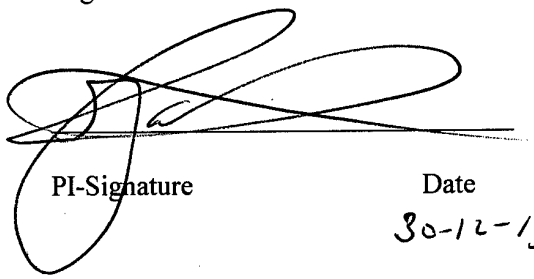

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30-12-1990

ACKNOWLEDGEMENTS

The authors are grateful to Helma E.T. Spruit, who developed the analytical procedures and measured the concentrations of sulfur mustard in the biological samples; to Willem C. Kuijpers, who performed the animal experiments; to Henk C. Trap for the development of the apparatus for nose-only and percutaneous exposures, for performing the respiratory and percutaneous exposures and for his contribution to the development of the gas chromatographic configurations; to John P. Oostdijk for development of the large volume gas chromatographic configuration; to Roos H. Mars-Groenendijk and Helma C.M. van Dijk-Knijnenburg for the analysis of 7-SM-gua; to Herman P.M. van Helden for supervising the animal experiments; to Cees van Hooidonk and Martine Polhuijs for the quality assurance within the project; to Herma J. van der Wiel for her advice with respect to the animal experiments and processing of tissues; to Marijke M.A. Mol for her advice with respect to the toxicologically relevant concentrations of sulfur mustard; to Brenda J. Lander, Maaike Husson, Sandy E.P.M. Roest and Johan Buurman for their contibution to the measurement of sulfur mustard in tissues, to Harry A. Versteegh for technical support; and to Martin C. Neeleman and Priscilla Vink for taking good care of the hairless guinea pigs. Furthermore, the authors wish to thank Dr. Hans-Gerd Janssen of the Technical University Eindhoven (The Netherlands) for the opportunity he offered us to test the sulfur chemiluminescence detector.

SUMMARY

The rapid proliferation of chemical weapons, the confirmed use of sulfur mustard (SM) in the Iran-Iraq conflict, the threat of chemical warfare in the Gulf War, and the recent terroristic attack with nerve agent in the Tokyo subway have stressed the need for reliable methods to detect nature and extent of poisoning with chemical warfare agents. Furthermore, adequate treatment for casualties of chemical agents, in particular of SM, is needed.
While the causes and treatment of skin damage due to SM are now being systematically investigated, the options for treatment of systemic intoxications are rather limited. The development of a therapeutic strategy requires insight into the behaviour of SM *in vivo*. Our extensive studies on the toxicokinetics of soman and sarin supported by Grants DAMD17-85-G-5004, DAMD17-87-G-7015, and DAMD17-90-Z-0034, have provided a quantitative basis for pretreatment and therapy of intoxications with nerve agents. By analogy, we postulate that knowledge of the toxicokinetics of intact SM can provide a quantitative basis for further toxicological investigations, as well as for (pre)treatment of intoxications with this vesicant. Toxicokinetic studies of SM have been very scarce so far. Moreover, these studies were hampered by a lack of verification of the analytical procedures, and by insufficiently low minimum detectable concentrations. We proposed to develop and verify analytical procedures for SM involving two-dimensional gas chromatography with thermodesorption/cold trap injection, at an estimated minimum detectable concentration of 5 pg SM/ml blood or g tissue. Based on these procedures we proposed to perform toxicokinetic investigations of SM in hairless guinea pigs after intravenous administration, as well as after respiratory and percutaneous exposure at militarily relevant doses corresponding with 0.3-1.0 LD50 or LCt50. We chose hairless guinea pigs for our experiments because of their usefulness for percutaneous exposure and protection experiments.
In close cooperation with the former TNO Medical Biological Laboratory, we developed within the context of Grants DAMD17-88-Z-8022 and DAMD17-92-V-2005 an ELISA based on a monoclonal antibody against the major adduct of SM with DNA (7-SM-gua), which detects $\geq$ 0.15 pmoles of this adduct/g tissue. We proposed to analyze these adducts in white blood cells, hematopoietic and other tissues, in conjunction with the toxicokinetic investigations of SM. This will provide unique insight into the *in vivo* relationship between the blood levels of intact SM and the resulting damage, expressed as concentration of SM-DNA adducts. Moreover, objective and quantitative procedures will be developed to establish damage in epithelial tissues, i.e., epidermis and corneal epithelium, at various Ct-values of SM.

In the 4-year cooperative agreement period, the following topics were studied in hairless guinea pigs:

(i) The conditions for stabilization and work-up of SM in biological samples; quantitative analysis in biological samples at SM-levels $\geq$ 5 pg/ml, as well as validation and automation of the analytical procedures;

(ii) The toxicokinetics of SM after intravenous administration of doses corresponding with 1.0 and 0.3 LD50;

(iii) The toxicokinetics of SM during and after 'nose-only' respiratory exposure to a dose corresponding with 1.0 and 3 LCt50;

(iv) The toxicokinetics of SM after percutaneous whole body exposure, with the animal breathing clean air, to SM vapor at a dose corresponding with *ca.* 1.0 LCt50, estimated from percutaneous toxicity data in the literature;

(v) In conjunction with the toxicokinetic experiments mentioned in (ii)-(iv), the concentrations of 7-SM-gua in the DNA of white blood cells, bone marrow, spleen, and either liver, small intestines or lung were determined during the period of formation and repair of this adduct;

(vi) In conjunction with the inhalation experiments of SM, the concentrations of 7-SM-gua in the DNA of the mucosa of various parts of the naso-pharyngeal airway and in other

parts of the respiratory tract were determined, in order to localize the areas of the respiratory tract where SM is absorbed and causes damage;

(vii) Using whole body exposure, a calibration curve was constructed relating skin damage expressed as amount of 7-SM-gua per 10^7 nucleotides in the epidermis and the corresponding 'Draize score' to Ct-values of SM in the range of 10-3000 mg.min.m^{-3}. In the same experiment DNA-adducts were measured in corneal epithelium, in order to quantify eye damage induced by exposure to SM vapor;

(viii) Using the calibration curve mentioned under (vii), the degree of protection afforded by two selected topical skin protectants was determined by comparing the concentrations of 7-SM-gua in the epidermis of the exposed skin areas, with and without application of the topical skin protectant at specific challenges of SM in liquid and vapor form;

(ix) Measurement of the effect of administration of the scavenger N-acetyl cysteine on the intravenous and respiratory toxicokinetics of SM and 7-SM-gua as well as similar measurements with an ester of cysteine as scavenger, upon respiratory exposure to SM.

A highly sensitive method for bioanalysis of intact SM in blood and tissues was developed, involving gas chromatography with automated thermodesorption injection and mass-spectrometric detection. Perdeuterated sulfur mustard was used as the internal standard. The absolute detection limit was 700 fg for sulfur mustard, which corresponded with a detection limit in blood of *ca.* 5 pg/ml. Concomitantly, gas chromatography with manual thermodesorption injection and pulsed-flame photometric detection was used for samples containing relatively high concetrations of SM. The detection limit of this procedure was *ca.* 125 pg/ml.
DNA-adducts were measured via the previously developed immunoslotblot method, using antibodies directed against the adduct of SM to guanine (7-SM-gua) in DNA.The detection limit of this procedure ranged between 3-13 7-SM-gua per 10^9 nucleotides.

The intravenous 96-h LD50 of sulfur mustard in the hairless guinea pig was determined and appeared to be 8.2 mg/kg (95 % confidence interval 7.1-8.8 mg/kg). The intravenous toxicokinetics of this dose in the hairless guinea pig are characterized by a very rapid distribution phase and a very slow elimination phase. The concentration of SM in tissues (lung, spleen, liver, and bone marrow) exceeded that in blood shortly after i.v. administration of the agent, indicating a considerable partitioning of SM from blood into the tissues. A rapid adduct formation occurred in blood and lung, and subsequently in other tissues. A considerable repair of the adducts was observed within 6 h. However, at 2 days after administration of SM, adducts were still detectable in most of the tissues studied.
The i.v. toxicokinetics of a dose corresponding with 0.3 LD50 showed the same general pattern. Non-linearity of the toxicokinetics with the dose was observed. The highest 7-SM-gua level was found in the blood. Adduct and intact sulfur mustard concentrations in the tissues were much lower than after i.v. administration of a dose corresponding with 1 LD50.

Next, the respiratory toxicokinetics were studied. The 96-h LCt50 of SM for 5-min nose-only exposure of hairless guinea pigs was determined to be 800 mg.min.m^{-3} (95 % confidence interval 700-920 mg.min.m^{-3}). During and after exposure of animals to 1 LCt50 SM the intact agent could not be detected in blood. 7-SM-gua was only detectable in the lung, at low concentrations. SM was measurable in the blood during and after nose-only exposure of hairless guinea pigs to 3 LCt50 of the toxicant. However, toxicokinetic evaluation of the concentration-time profile was rather complicated, and was only possible by assuming the existence of two absorption processes. Measurable concentrations of 7-SM-gua were found in the blood and lungs of these animals, whereas marginal adduct levels were found in the spleen.
The respiratory tract was isolated from animals nose-only exposed to 1 LCt50 SM in 5 min, at 4 h after ending the exposure. The tract was divided into 6 regions. Most adduct formation had occurred in the larynx and trachea, whereas hardly any 7-SM-gua was found in the lung. The

observation that SM was not detected in the blood of animals exposed to 1 LCt50 SM may be explained from the distribution of the agent within the respiratory tract.
Furthermore, a histopathological evaluation of the 6 regions of the respiratory tract of the exposed animals was performed, which showed substantial damage in mainly the upper airways.

Next, the percutaneous toxicokinetics of SM in the hairless guinea pig were studied . During and after a *ca.* 45-min percutaneous exposure of the animals to a Ct of 10,000 mg.min.m^{-3}, concentrations of SM in blood up to *ca.* 12 ng/ml could be detected. The concentration-time profile of SM in blood during and after percutaneous exposure resembled that of the nose-only exposure to a Ct of 2,400 mg.min.m^{-3}: the existence of a rapid and a slow absorption process is suspected. Shortly after ending the exposure concentrations of intact SM exceeding that in blood were measured in the tissues. Rather low concentrations of 7-SM-gua were measured in most tissues. From most of the skin samples, DNA could not be isolated, indicating massive DNA damage which leads to cross-linking between DNA and proteins.
From the percutaneous toxicokinetic experiments it can be concluded that systemic intoxication form SM is more likely to occur from a percutaneous exposure than from a respiratory exposure.

The protection of hairless guinea pigs against the respiratory toxicity of SM by two scavengers, i.e., N-acetyl cysteine (NAC) and cysteine isopropylester (CIPE) was determined. Animals were pretreated with 5 mmoles of scavenger per kg bodyweight, 1 min prior to the respiratory challenge with SM mustard. The 96-h LCt50 of sulfur mustard in NAC-pretreated animals was 1285 mg.min.m^{-3} (95 % confidence interval 770-2110 mg.min.m^{-3}) and 1125 mg.min.m^{-3} (95-% confidence interval 665-1275 mg.min.m^{-3}) for CIPE-pretreated animals, which is not significantly higher than the LCt50 in non-pretreated animals.

Topical Skin Protectants 1511 and 2701 protected hairless guinea pigs against skin damage caused by liquid SM and SM vapor. Based on the amount of DNA adducts in skin, the protection against a vapor challenge appeared to be almost complete, whereas protection against liquid SM was somewhat less effective. Whereas the TSP's performed equally well against SM vapor, TSP 2701 performed better against liquid SM than TSP 1511.

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I. INTRODUCTION

The treatment of sulfur mustard (SM) casualties from the Iran-Iraq war has provided ample evidence that a specific and causal therapy for the local and systemic effects of this vesicant is not available (Willems, 1989). In fact, treatment had to be restricted to sustaining the vital functions of the patient, enhancing the healing process of the lesions and preventing secondary infections. Evidently, research on the toxicology of SM has been neglected since the end of World War II, when the threat of the newly developed nerve agents became apparent. However, the use of SM in the Iran-Iraq war has reconfirmed the threat of vesicants on the battlefield. Since several countries have provided themselves with stocks of this agent, great efforts have to be made in order to develop a better treatment of SM intoxication. This is true for the local effects on skin, eyes and respiratory tract, as well as for the systemic effects (Papirmeister *et al.*, 1991). In general it may be stated that the local effects of SM are responsible for turning a soldier into a casualty whereas the duration of his incapacitation, and of his burden on the medical staff, is determined by the systemic effects of the agent (Stade, 1964). Efforts in the field of diagnosis and dosimetry of SM exposure (Benschop, 1991) and with regard to treatment of its effects on skin are well under way (Papirmeister *et al.*, 1991). However, relatively little effort has gone into the prophylaxis and/or therapy of systemic intoxication with the agent. In virtue of its large penetrative capacity and extensive biochemical activity, SM is transported rapidly by skin absorption and inhalation to various organs, where it exerts its effects. It affects, amongst others, the bone marrow, liver, kidneys, stomach and intestinal tract, as well as the central nervous system and the metabolic pathways in general. A variety of symptoms may occur, such as general malaise, apathy and deep depression. These symptoms are, to some extent, even more insufferable than the vesicant effects. Moreover, it has been well established that victims suffer from a variety of chronic and delayed effects for the rest of their lifetime, such as asthma, bronchitis, premature ageing, loss of libido and potency, central nervous system effects and cancers of the respiratory tract (SIPRI, 1975).

The only positive results to prevent or diminish the effects of systemic intoxication with SM have been obtained with scavengers having a high reactivity for the episulfonium derivative of the agent, e.g., thiosulfate which is active in the extracellular space, or cysteine and other thiol derivatives which may penetrate the cell (Connors, 1966; Callaway and Pearce, 1958). High doses of thiosulfate or cysteine given shortly before or after intoxication afford some protection against the lethality of SM in experimental animals, but the effects of thiosulfate on nonlethal damage have hardly been investigated (Vojvodic *et al.*, 1985). Recent investigations concentrate on the scavenger activity of N-acetyl cysteine (Trouiller and Lainee, 1992; Anari *et al.*, 1988) and on cysteine alkyl esters which are enzymatically hydrolyzed within the cell (Upshall and Lawston, 1991).

Efforts to develop a causal treatment of systemic intoxication with SM, or with any toxic agent in general, require an intimate knowledge of the toxicokinetics of the intact agent as well as insight into the etiology of the lesions which are of major importance in the development of systemic intoxication. In the case of intoxication with nerve agents, our detailed toxicokinetic investigations of the agents sarin and soman in several species including primates have provided a quantitative basis for further toxicological research of this agent and for development of strategies for treatment or pretreatment of intoxication (Benschop and De Jong, 1990, 1991; Langenberg *et al.*, 1996). By analogy, we expect that similar toxicokinetic studies of intact SM will provide a quantitative basis for causal treatment of systemic intoxication, in particular with scavengers. Toxicokinetic studies of SM including distribution of the agent in various tissues will also provide a starting point for the development of a physiologically based toxicokinetic model, which enables interspecies scaling and, eventually, extrapolation to man. Such a model is currently being developed for soman toxicokinetics (Langenberg *et al.*, 1997a).

In current theories on the etiology of local and systemic damage due to exposure to SM, it has been hypothesized that alkylation of DNA is often the starting point for a chain of reactions leading to the observed damage (Papirmeister *et al.*, 1991) although current interest is focused

on the effects of direct alkylation of proteins. Therefore, the amount of SM adducts of DNA and proteins in a tissue can be regarded as a measure of the amount of damage in that tissue. Since we have developed several methods to measure the amount of DNA-adducts of SM in our investigations on diagnosis and dosimetry of exposure to SM (Benschop, 1991), we proposed to combine the toxicokinetic investigations of intact SM in blood and selected organs with the analysis of DNA-adducts in these tissues, in order to obtain insight into the relationship between the challenge and the damage due to that challenge.

Until recently, studies on the *in vivo* distribution of SM were performed mostly with radiolabeled SM, which leaves in doubt whether results pertain to intact agent or to hydrolyzed and metabolized derivatives (Black *et al.*, 1992). Moreover, these studies involved mostly intravenous administration whereas absorption via the respiratory tract and the skin are the military relevant portes d'entrée for systemic intoxication. Investigations with intravenous administration of ^{35}S-SM in mice (Clemedson *et al.*, 1963) and in rabbits (Boursnell *et al.*, 1946) indicate a rapid and rather even distribution over the body with some accumulation appearing in the nasal region, kidneys, liver, lungs and intestines. Upon percutaneous administration of liquid SM to mice (Clemedson *et al.*, 1963), a slower clearance was observed, as expected from gradual uptake through the skin. Assessment of the damage in the respiratory tract and in the lungs after inhalation of SM suggests that most of the agent is absorbed before it reaches the lungs (Cameron *et al.*, 1946).

Recently, two groups have reported results on the toxicokinetics of intact SM. Zhang and Wu (1987) measured blood levels of SM after intravenous, subcutaneous, and percutaneous administration of SM to piglets. Using gas chromatography on packed columns and flame-photometric detection they were able to follow the blood levels of intact SM for 20 min after intravenous administration of 10 mg SM/kg and for 90 min after subcutaneous administration of 200 mg SM/kg. Intact SM could not be observed after percutaneous application of 200 mg liquid SM/kg on a 5 cm^2 skin area. However, their apparent lower detection limit is in the range of 200 ng SM/ml blood, which should be considered as approximately *ca.* 3 orders of magnitude too high for toxicologically relevant analysis of such highly reactive agents. Maisonneuve *et al.* (1993) measured blood levels of intact SM in rats after i.v. administration of 10 mg SM/kg, corresponding with *ca.* 3 LD50. Using gas chromatography with capillary columns and flame ionisation detection, they obtained a detection limit of *ca.* 10 ng SM/ml blood, which allowed them to follow blood levels of intact SM for 8 h. These authors, as well as Zhang and Wu (1987), observed a rapid distribution of the agent with a half-life of *ca.* 5.6 min, followed by slow elimination with a half-life of 3.6 h. The observed blood levels in piglets and rats after administration of multiple lethal doses of SM cast extra doubt on the colorimetrically measured levels of intact SM in blood (1.1 µg/ml) and tissues of an Iranian patient who died 7 days after exposure to SM (Drasch *et al.*, 1987).

In summary, these preliminary investigations on the toxicokinetics of SM show that, as in the case of nerve agents, intact SM has a much longer *in vivo* lifetime than assumed previously on the basis of rapid hydrolysis of SM in aqueous solution. When this persistence is confirmed in our proposed investigations, these results will have to be integrated into future investigations on the toxicology of SM and on treatment of intoxications with the agent.

On the basis of the considerations mentioned above, we proposed investigations for a 3-year Cooperative Agreement on the toxicokinetics of SM in the hairless guinea pig. In the following the various aspects of the proposed research are further elucidated.

(a) Stabilization, work-up and analysis of SM in biological samples.

Our experience with the analysis of nerve agents in biological samples (*cf.* Benschop *et al.*, 1985) suggested that immediate stabilization of SM in blood and tissue samples would be crucial, in view of the high reactivity of the agent towards the biological matrix. The influence of parameters such as pH, temperature and concentration of chloride ions was studied. The analysis of known concentrations of SM spiked into stabilized blood samples would show

whether the stabilization procedure was adequate. It was investigated whether solid-liquid extraction with C_{18}- or other cartridges improved the work-up of samples in terms of preconcentration for gas chromatographic analysis, removal of contaminants, and convenience in comparison with previously applied liquid-liquid extraction.
For final analysis of SM we relied on the gas chromatographic configuration which has provided excellent results with nerve agent analysis, i.e., thermal desorption (from Tenax)/cold trap (TCT) injection and two-dimensional chromatography (Benschop and De Jong, 1990). For detection we intended to use a flamephotometric, mass spectrometric, chemoluminescence or electron capture detector. It was attempted to use fully deuterated SM, i.e., D_8-SM as an internal standard. This configuration assured superior "on-line work-up" and selectivity. Also, it was be attempted to automate the TCT-injection in order to increase sample throughput. With this analytical ensemble we anticipated a detection limit of *ca.* 5 pg SM/ml blood, which we hoped would be sufficient for the purpose of this study.

(b) The hairless guinea pig as selected species
The hairless guinea pig is used extensively in studies on skin damage by SM and of skin protectants since the structural effects of SM on the cells in the basal layer of skin are nearly identical to those in human skin (Papirmeister *et al.*, 1991). The epidermis in the hairless guinea pig is much thicker than in other laboratory animals, i.e., more similar to that in humans (Mershon *et al.*, 1990). Therefore, this species allows highly relevant investigations on skin damage, skin protection, and systemic absorption upon exposure to SM vapor.
The hairless guinea pig was also used in this study for toxicokinetic investigations following intravenous administration and respiratory exposure.

(c) Doses and routes of administration of SM
Experience with the use of SM on the battlefield has learned that this agent should be characterized as a physical incapacitating agent rather than as a lethal agent. In World War I, only *ca.* 2 % of SM casualties died, often due to secondary infections resulting from damage to the respiratory tract (Papirmeister *et al.*, 1991). Therefore, we proposed to use doses of SM which are equivalent to 1.0 LD50 or 1.0 LCt50 as the highest dose. In cases where the influence of dose on the toxicokinetics is investigated we proposed to use an additional dose corresponding with 0.3 LD50 or LCt50. However, if the latter dose would not lead to measurable blood levels of SM, it would be replaced by a tenfold higher dose.

(i) Intravenous administration of SM
In order to obtain insight into the basic aspects of the toxicokinetics of SM in hairless guinea pigs, the investigations were initiated by measuring the concentration-time course of SM in blood after intravenous administration of the agent at doses corresponding with 1.0 and 0.3 LD50. This provided data on the blood levels of intact SM that could be expected in further experiments, on the (non)linearity of the toxicokinetics with dose, as well as on the time scale in which detectable concentrations could be measured.
Furthermore, concentrations of intact SM were measured in selected tissues at various time points after intravenous administration of the toxicant, in order to obtain insight into the distribution of SM within the body.

(ii) Respiratory exposure to SM
It is generally accepted that absorption of SM vapor in the respiratory tract and via the skin are the military relevant portes d'entrée for this agent. Therefore, further toxicokinetic experiments pertained to these routes of exposure.
A new apparatus was designed for respiratory exposure of hairless guinea pigs, using the experiences obtained in our inhalation toxicokinetic studies on the nerve agents soman and sarin (Benschop and Van Helden, 1993). We measured the concentration-time course of SM in blood of hairless guinea pigs after 'nose-only' exposure for 5 min to a dose of SM corresponding with

1.0 LCt50. In order to measure the degree of linearity of the toxicokinetics with exposure dose, the same experiment was performed at a 'dose' corresponding with 0.3 LCt50.
Furthermore, concentrations of intact SM were measured in selected tissues at various time points after ending the nose-only exposure to the toxicant, in order to obtain insight into the distribution of SM within the body after respiratory exposure.

(iii) *Percutaneous exposure to SM*
Exposure of the skin to SM vapor is known to lead to skin damage as well as to systemic intoxication. The blood concentration-time course of SM was measured during and after a 35-min whole body exposure of hairless guinea pigs to the estimated percutaneous LCt50 of SM in various species, i.e., 10.000 $mg.min.m^{-3}$ (Papirmeister *et al.*, 1991). During these exposures, the animals breathed clean air. An exposure chamber was designed and constructed for these purposes.
Furthermore, concentrations of intact SM were measured in selected tissues at various time points after ending the percutaneous exposure to the toxicant, in order to obtain insight into the distribution of SM within the body.

(d) Quantitative analysis of SM-DNA adducts in blood and various tissues
As mentioned above, it is assumed that the concentration of SM-DNA adducts in a tissue is a reasonable measure for the damage due to SM in that tissue. We have developed an ELISA based on a monoclonal antibody against the major SM-adduct with DNA, i.e., the monoadduct at the 7-position of guanine (7-SM-gua). This ELISA detects one such adduct amongst $2*10^7$ nucleotides in DNA, i.e., $\geq$ 0.15 pmol of adduct/g tissue (Benschop and Van der Schans, 1995). We proposed to combine our toxicokinetic investigations of intact SM in blood with measurements of the formation of 7-SM-gua in blood and in selected tissues, concomittantly with analysis of SM in these tissues. In this way, insight is obtained into the relationship between the concentrations of SM in blood and these tissues and the concentration of the resulting DNA adducts. Also, the repair of the adducts with time was followed. Similar integrated investigations on toxicokinetics of direct or metabolically formed alkylating agents and of resulting DNA-adducts have been rarely performed (see for example Ginsberg and Atherholt, 1990) and may have general usefulness for studies on carcinogenesis.

The selection of cell types and tissues for analysis of SM-DNA adducts was not straightforward, since SM appears to be rather unselective in its systemic toxicity. The following cell types and tissues were proposed for initial measurement of SM-DNA adducts:

- *white blood cells*, being primary targets and indicators for exposure to SM, no matter how the agent reaches the general circulation.

- *bone marrow* and *spleen*, since in all systemic intoxications with SM, damage to the bone marrow and the hematopoietic system in general are the most striking effects (Needham *et al.*, 1947). Leucopenia is the main result of the damage; the red cell system is much less affected.

- *(small) intestine*, since the so-called radiomimetic effects of SM relate primarily to damage to the small intestine.

- *lungs*, being often invoked as damaged tissues in systemic intoxications with SM.

- *liver*, because of its crucial role in general metabolism.

We proposed to investigate in some detail the formation and repair of 7-SM-gua adducts in DNA of the above-mentioned tissues upon intravenous administration of 1 LD50 of SM to hairless guinea pigs. It was investigated on which time scale the maximal concentrations of

adducts are formed. Based on the results of Ginsberg and Atherholt (1990), it was assumed that the adducts are formed within the first half hour after intravenous administration or respiratory exposure. In addition, the time scale of formation of the adducts was investigated separately upon percutaneous exposure since we anticipated that the adducts would be formed more slowly in this case. In view of our data on the *in vivo* repair of SM-DNA adducts in skin (Benschop and Van der Schans, 1995), the repair of SM-DNA adducts in the abovementioned tissues was investigated at 24 and 48 h after intravenous administration.
Based on the exploratory results after intravenous administration and respiratory and percutaneous exposure, the time points for analysis of formation and repair of 7-SM-gua, as well as of SM, were determined for the remaining toxicokinetic experiments. The adduct was measured in white blood cells, bone marrow and spleen. In addition, 7-SM-gua was measured in either lungs, liver, or small intestines, whichever of these tissues appeared to accumulate the highest concentration of adduct upon intravenous administration of SM.

(e) Absorption of SM in the nasal-pharyngeal airway and respiratory tract
Cameron *et al.* (1946) mention the following concise observation: "When animals inhale lethal concentrations of mustard gas [or nitrogen-mustard] vapour, death results from direct damage to the respiratory tract with or without systemic poisoning. With certain small species, however (rabbit, guinea pig and rat), death from systemic absorption is frequently observed with little or no damage in the respiratory tract, apart from severe inflammation of the nose, which is always present. It appears therefore that a lethal dose of certain vapours may be absorbed through the mucous membrane of the nose". These authors concluded from their experiments that rabbits absorb *ca.* 80 % of inhaled SM vapor in the nose. Our proposed experiments dealing with the inhalation toxicokinetics of SM at a dose corresponding with 1.0 LCt50 in guinea pigs (*cf.* c(ii)), and our ability to measure the major DNA-adduct of SM in various tissues (d), offer a unique possibility to combine the investigation of systemic absorption with the measurement of DNA adducts in various parts of the nasal-pharyngeal airway and the further respiratory tract.
Assuming that the concentration of DNA-adducts in a certain area of the respiratory system is proportional to the concentration of SM vapor that is encountered (Casanova *et al.*, 1991), these adducts were measured in the mucous membranes of the middle turbinates, anterior lateral walls and septum, nasopharynx, maxillary sinuses, larynx-trachea-carina, major interpulmonary airways, and lung. In combination with a histopathological investigation of the damage in the respiratory tract, detailed information was obtained on the sites of absorption in the respiratory tract, in conjunction with observed damage.

(f) Quantitation of skin damage based on SM-DNA adducts in the epidermis
In order to assess skin damage due to exposure to SM, the so-called Draize test is often used. This test depends on the evaluation of the degree of skin erythema by way of a rather subjective visual assessment (Papirmeister *et al.*, 1991). A priori, the procedures that we developed for analysis of 7-SM-gua in DNA of blood and various tissues (*cf.* item d) can be used to assess skin damage in a far more quantitative and objective way, taking also into account that the amount of DNA adducts is proportional to the amount of protein adducts (Skipper *et al.*, 1994).
Based on this reasoning we proposed the following experiments in order to develop an objective test for skin damage due to exposure to SM.
Using whole body exposure, hairless guinea pigs were exposed for a 10-min period to various concentrations of SM-vapor in air to yield Ct-values of SM ranging between 100 and 3000 $mg.min.m^{-3}$. These Ct-values are likely to cause slight erythema at the lower Ct-range up to very severe skin damage at the higher Ct-range (Papirmeister *et al.*, 1991). The amount of 7-SM-gua was analyzed immediately after exposure according to the procedure mentioned under item (d) in the epidermal layer from skin biopts.
The skin damage was also assessed according to the Draize procedure at 24 h after exposure, in order to correlate results with the new procedure.

(g) Quantitation of eye damage based on SM-DNA adducts in corneal epithelium

It is well known that the human eye is extremely sensitive to exposure to SM in vapor or liquid form. A Ct-value of 10 $mg.min.m^{-3}$ will already cause reddening of the eye. Exposure to higher Ct-values leads to conjunctivitis, photophobia, corneal edema and clouding (Papirmeister *et al.*, 1991; Grant, 1986; Duke-Elder, 1954). Tests used to estimate damage due to SM exposure of the eye are very indirect, by way of measurement of loosening of corneal epithelium (Hermann and Hickman, 1948). We proposed to assess eye damage analogous to skin damage. Eyes from the same hairless guinea pigs as used for the exposures described in item (f) were enucleated and the amount of 7-SM-gua was determined in corneal epithelium. Hence, data became available which allow a straightforward, sensitive and objective test for eye damage. Moreover, a comparison of the concentrations of SM-DNA adduct in epidermis and in corneal epithelium from the same animal allows a comparison of the relative susceptibility of these two epithelial tissues to damage caused by exposure to SM. Similar experiments have been performed with regard to UV-damage in these two types of tissue (Freeman *et al.*, 1988; Ley *et al.*, 1988).

(h) Quantitation of the protective efficacy of topical skin protectants based on SM-DNA adducts in the epidermis

Topical skin protectants (TSP's) are being developed for use in cases where military personnel will have to perform tasks in an environment contaminated by SM, without being fully protected by means of protective clothing. In such special circumstances, contamination of the skin by liquid as well as vapor of SM might be a realistic threat. Therefore, we proposed to test the protective efficacy of two TSP's (selected by the U.S. Army Medical Research Institute of Chemical Defense), as measured by the reduction in the concentration of 7-SM-gua in the epidermis due to the application of the TSP.

According to the procedure of Mershon *et al.* (1990), skin exposure sites (*ca.* 1 cm^2) on hairless guinea pigs were covered with a topical skin protectant. Seven minutes after application of 1 µl of liquid SM on these covered sites, the sites were decontaminated and the amount of 7-SM-gua in the epidermis of the exposed site was analyzed, two hours after ending the exposure. On the same animal, comparable unprotected and covered sites were challenged for the same period of time with the same amount of liquid SM. After subsequent decontamination and analysis of adduct in the epidermis, a comparison of the amount of adduct in the protected and unprotected sites provided a 'protective ratio' for the TSP.

Although TSP's are primarily being developed for protection against liquid SM, these protectants were also tested for their efficacy against SM vapor. Skin sites on hairless guinea pigs protected by TSP as described above were challenged for 10 min with a Ct of SM vapor that would yield severe skin damage, according to the results of the experiments described under item (f). Subsequently, the amount of 7-SM-gua in the epidermis of the protected skin site was determined as mentioned above. A comparison with the results obtained in experiments described under item (f) provided a 'protective ratio' for the TSP against SM vapor.

(i) Influence of scavengers on the intravenous and inhalation toxicokinetics of SM and SM-DNA adducts

As mentioned by Papirmeister *et al.* (1991), administration of sodium thiosulfate at high doses has consistently been found to provide some protection from the systemic toxicity of SM. This effect has been related to the high competition factor of thiosulfate relative to water in its reaction with the episulfonium derivative of SM (Ogston *et al.*, 1948). For example, Vojvodic *et al.* (1985) showed that sodium thiosulfate, administered intraperitoneally at a dose of 3 g/kg at 30 min after subcutaneous poisoning with SM in rats (i) decreased lethality resulting in a protective ratio of 1.7, (ii) prolonged survival time, (iii) antagonized decrease in body weight, and (iv) lessened the degree of histopathologic damage to various tissues, e.g., spleen and liver. In view of the extremely high doses of thiosulfate which are needed to provide a protective effect, the use of this scavenger as a (pre)treatment agent is questionable. Moreover, it is known that thiosulfate does not penetrate into the intracellular space. Therefore, recent investigations

on potential scavengers have concentrated on thiol derivatives which can penetrate the cell and are active at lower doses than thiosulfate. Iranian investigators (Anari *et al.*, 1988) have published a paper on the efficacy of N-acetyl cysteine in experimental SM poisoning. Their results have been confirmed (Trouiller and Lainée, 1992). This compound enters the cell and has proven its value in a variety of intoxications in which highly electrophilic species such as the episulfonium ion should be scavenged. For example, N-acetyl cysteine is used frequently, at an intravenous dose of *ca.* 300 mg/kg, in paracetamol intoxications (Prescott *et al.*, 1979). Upshall and Lawston (1991) have filed a patent application dealing with cysteine esters as scavengers for electrophilic compounds, e.g., perfluoroisobutylene and SM. These compounds also enter the cell, where they are hydrolyzed enzymatically to cysteine, especially in the lungs.

In view of the supposed scavenging effect of N-acetyl cysteine and of cysteine esters, it should be expected that administration of these scavengers shortly before or after systemic intoxication with SM, should decrease the area under the curve for the blood levels of SM, as well as the concentrations of SM-DNA adducts in blood and in various tissues. In order to investigate the scavenging effect on the blood levels of SM, we propose to investigate the protective efficacy of intraperitoneal administration of N-acetyl cysteine and of a cysteine ester 1 min before nose-only exposure of hairless guinea pigs for 5 min to various concentrations of SM vapor.
If these scavengers show a promising antidotal efficacy against SM, we proposed to

(i) analyze the concentrations of SM, 7-SM-gua, and of N-acetyl cysteine in blood and in several tissues at various time points after intravenous administration of SM to hairless guinea pigs at a dose corresponding with 1 LD50, with intraperitoneal administration of N-acetyl cysteine at 1 min before intoxication;

(ii) perform similar analyses as described in (i) upon nose-only exposure of hairless guinea pigs for 5 min to SM vapor to yield a Ct corresponding with 1 LCt50, with intraperitoneal administration of N-acetyl cysteine 1 min before exposure to SM vapor;

(iii) perform experiments and analyses similar to those described in (ii) (except for scavenger levels) using a cysteine alkyl ester as scavenger, which will be administered intraperitoneally 1 min before exposure to SM.

(iv) attempt a description of the effect of N-acetyl cysteine on the blood levels of SM in a toxicokinetic model (*cf.* Fast and Sorbo, 1973).

The abovementioned investigations were intended to serve as a starting point for quantitative investigations on the effect of scavengers on systemic intoxication with SM. If modelling would be succesful this would allow the prediction of the effect of reactivity, affinity of antibodies (Lieske *et al.*, 1992), doses, and methods of administration (e.g., continuous infusion, *cf.* Hatea, 1986) on the efficacy of scavengers.

Due to the problematic availability of hairless guinea pig, the performance period of the cooperative agreement was extended from 3 years to 4 years and 3 months. In this Final Report all obtained results are described and discussed.

II. EXPERIMENTAL PROCEDURES

II.1. Materials

WARNING: Sulfur mustard is a primary carcinogenic, vesicating, and cytotoxic agent. This compound should be handled only in fume hoods by experienced personnel.

Technical grade sulfur mustard (SM) was purified by fractional distillation in a cracking tube column (Fischer, Meckenheim, Germany) to a gas chromatographic purity exceeding 99.5 %. The internal standard, D_8-sulfur mustard (D_8-SM) was obtained as described elsewhere (Benschop and Van der Schans, 1995). Ethyl acetate ('zur Rückstandsanalyse') was procured from Merck (Darmstadt, Germany) and was distilled over a column packed with Dixon rings (plate number 80; NGW, Wertheim, Germany) before use. Isopropanol (purity > 99.5 %) was purchased from Fluka (Buchs, Switzerland).
The following products were obtained commercially and were used without further purification: heparin (Vitrum, Stockholm, 5000 IU/ml), ketamine hydrochloride (Vetalar®, Parke-Davis, Morris Plains, NJ, USA), buprenorfine hydrochloride (Temgesic®, Schering-Plough, Amstelveen, The Netherlands), HPLC-grade water (Fisons, Loughborough, UK), Tenax TA, 60-80 mesh (Chrompack, Middelburg, The Netherlands). Disodium edetate (EDTA), Triton® X-100, sodium chloride, potassium chloride, disodium hydrogen phosphate dihydrate, potassium dihydrogen phosphate, phenol, phosphoric acid (85 %), chloroform and ethanol were obtained from Merck (Darmstadt, Germany); sodium dodecyl sulfate (SDS), Tris.HCl from J.T. Baker (Phillipsburg, NJ, USA), and isoamyl alcohol from UCB (Brussels, Belgium); calf thymus DNA, proteinase K (EC 3.4.21.14, activity *ca.* 20 units/mg protein) and RNase T1 (EC3.1.27.5, activity *ca.* 40 units/mg protein) from Boehringer (Mannheim, Germany); RNase A, and Tween 20 from Sigma Chemical Co. (St. Louis, MO, USA); and skimmed milk powder, less than 1 % fat, from Campina (Eindhoven, The Netherlands).
N-acetyl cysteine was purchased as Fluimucil®.
Cysteine isopropyl ester (CIPE) was synthesized according to the procedure described by Lailey *et al.* (1991). Anhydrous hydrogen chloride (86.8 g) was passed into 2-propanol (685 ml) in 30 min. L-Cysteine (46.4 g) was added and the mixture was heated under reflux for 4 h. At atmospheric pressure *ca.* 400 ml of 2-propanol was distilled in 1 h. After hot filtration of the residue, the desired product crystallized in the cooled filtrate. After filtration the product was dried overnight at 35 °C under reduced pressure. About 30.5 g of CIPE hydrochloride was obtained, which corresponds with a yield of *ca.* 40 %. The melting point was determined to be 155-157 °C (literature 155-156 °C). The identity of the compound was confirmed with ^{1}H- and ^{13}C-NMR. According to NMR analysis, the product contained *ca.* 95 % CIPE hydrochloride.

Topical skin protectants 1511 and 2701 were obtained from Dr. Ernest H. Braue of the US Army Medical Research Institute of Chemical Defense (Aberdeen Proving Ground, MD, USA). Lot numbers were not provided.
Immunoslotblot assays were carried out with Schleicher & Schuell minifold S (6 mm^2 slots; Schleicher & Schuell, Dassel, Germany) and nitrocellulose filters (pore size 0.1 µm; Schleicher and Schuell). DNA was immobilized by UV-crosslinking with a GS Gene Linker UV chamber (Bio-Rad Laboratories, Hercules, CA, USA). The enhanced chemiluminescence blotting detection system (Boehringer) was used for the detection of peroxidase activity. The developed film was scanned with a densitometer (Ultroscan XL, Pharmacia). In later experiments the chemiluminescence was recorded with a 1450 MicroBeta Trilux Luminescence Counter (EG & G Wallac, Breda, The Netherlands).

II.2 Gas chromatography

The gas chromatographic configurations used or developed for our investigations are described below. They will be referred to by number in this report.

GLC configuration 1 (GC-FID)
This configuration was used to study the extraction recovery of SM from blood. A Carlo Erba (Milano, Italy) HRGC 5160 Mega series gas chromatograph was equipped with an AS550 on-column autosampler and a flame ionization detector (FID). The analytical column was CPSil 5 CB (length, 30 m; i.d., 0.32 mm; film thickness, 1 μm), connected to the injector via a retention gap of uncoated deactivated fused silica (medium polarity; length, 1 m; i.d., 0.53 mm). Carrier gas (helium) pressure was set at 100 kPa. The oven temperature was programmed from 87 °C to 135 °C at a rate of 10 °C/min. The temperature was kept at 135 °C for 5 min, after which the oven was cooled to 87 °C for the next run. The temperature of the detector base was set at 250 °C. Flow rates of air and hydrogen through the FID were 350 and 35 ml/min, respectively. No make-up flow was needed.

GLC configuration 2 (LV-GC-GC-ECD, cf. Figure 1, subsection III.1.c)
This configuration was equipped for automated analysis of SM in extracts from blood, using large volume on column injection, two-dimensional chromatography and electron capture detection (ECD).
A Carlo Erba 5300 Mega series gas chromatograph was equipped with an electron capture detector (ECD) and a flame ionization detector (FID), a Waters WISP 590B (Millipore, Milford, MA, USA) HPLC autosampler and a Waters HPLC 590 programmable pump for micro flow pumping, a Valco (Schenkon, Switzerland) 8-port injection valve with electrical actuator and a Chrompack (Middelburg, The Netherlands) MUSIC (**Mu**ltiple **S**witching **I**ntelligent **C**ontroller) system. Flow rates of air and hydrogen through the FID were 350 and 35 ml/min, respectively. Nitrogen was used as make-up gas for the ECD, at a flow rate of *ca.* 40 ml/min. The temperature of the FID detector base was set at 250 °C, that of the ECD at 300 °C. The temperature of the ECD detector itself was set at 310 °C. The nitrogen pressure of the ECD was set at 110 kPa. The reference current was 1 mA, the pulse voltage was 5 V.
A small piece of deactivated uncoated fused silica (length, 50 cm; i.d., 0.10 mm) was used for transferring the analytes from the 8-port injection valve to the injector. The retention gap was an uncoated deactivated (medium polarity) fused silica column (length, 15 m; i.d., 0.53 mm). The retaining precolumn (length, 4m; i.d., 0.53 mm) was coated with chemically bonded CPSil 8 CB (film thickness, 5 μm), connected via a Y-shaped splitter to the 8-port valve. The precolumn in the MUSIC system (length, 10 m; i.d., 0.53 mm) was coated with chemically bonded CPSil 8 CB (film thickness, 5 μm). The analytical column for the analysis of SM and D_8-SM was a fused silica column (length, 33 m; i.d., 0.32 mm) coated with chemically bonded CPSil 19 CB (film thickness, 1μm). Small pieces of deactivated uncoated fused silica (i.d., 0.25 mm) were used as connecting material and trap intermediary in the MUSIC. A piece (length, 0.30 m; i.d., 0.25 mm) of deactivated uncoated fused silica column was mounted to the 8-port injection valve and used as a restriction capillary. All columns were purchased from Chrompack (Middelburg, The Netherlands). Connections between the various columns were made with glass press-fit connectors.
The HPLC pump operated at an ethyl acetate flow-rate of 185 μl/min. The injection volume of the autosampler was set at 400 μl. The dead volume between the autosampler and the end of the injection capillary was 57 μl. At a flow-rate of 185 μl/min this dead volume was passed in 0.31 min. During this time period the solvent passed through the 8-port valve to the 'waste'. After this period the valve was switched to the injection position, upon which the waste was closed and the 'early solvent vapor exit' (ESVE) was opened. During injection the ESVE was kept at 100 °C, to prevent condensation of the solvent in the valve. The solvent (ethyl acetate) evaporated and was vented, while the analytes were concentrated on the retaining precolumn. The time period for ESVE equalled the injection time of 2.26 min. A shorter time period could result in insufficient removal of the solvent, whereas a longer time period could result in loss of analyte. After the 2.26-min injection time the valve switched back, opening the waste, and closing the ESVE. The HPLC-pump stopped after 3 min, awaiting the next injection.

After the ESVE-step, the analytes were injected on the precolumn. The temperature program of the GC was as follows: 70 °C for 8 min, heating to 140 °C at 10 °C/min, 140 °C for 15 min, cooling to 70 °C at 'infinite' rate. The pressure of the carrier gas was set at 184 kPa. The fraction containing SM and the deuterated internal standard were trapped in the cold trap at -70 °C. The time at which trapping had to start and end, which was operated by the MUSIC controller, was determined by evaluating the precolumn chromatogram for which a flame ionization detector (FID) was connected to the precolumn. Ideally, SM and D_8-SM are not resolved on the precolumn, enabling trapping of a narrow fraction of the precolumn chromatogram.
Next, the trapped analytes were reinjected onto the analytical column, by flash-heating of the cold trap. Both the trap and trap-base temperature were set at 180 °C. Carrier gas pressure for the analytical column was set at 150 kPa.

GLC configuration 3 (GC-MS)
The GC-MS configuration consisted of a Carlo Erba HRGC 5300 gas chromatograph equipped with an AS 550 autosampler and connected to a quadruple mass-spectrometer (Automass 150, Unicam).
MS-detection was performed by positive electron impact ionization (70 keV) under full-scan conditions at m/z 50-200 for samples containing relatively high concentrations of SM, and under semi-single ion monitoring conditions, at m/z 109 for SM and 115 for D_8-SM for samples containing relatively low concentrations of SM, i.e., < 10 ng/ml.
The analytical column was CPSil 5 CBMS (length, 30 m; i.d., 0.25 mm; film thickness, 0.25 µm), connected to the injector via a retention gap of uncoated deactived fused silica (medium polarity; length, 2 m, i.d., 0.53 mm). Carrier gas (helium) pressure was set at 130 kPa. The oven temperature was programmed from 87 to 135 °C at a rate of 25 °C/min. The temperature was kept at 135 °C for 3 min, after which the oven was cooled to 87 °C for the next run. The interface temperature and the source temperature were set at 120 °C.

GLC configuration 4 (TDAS-GC-MS)
This configuration resembles GLC configuration 3, albeit that the AS550 autosampler was replaced with a thermodesorption autosampler (TDAS, Carlo Erba). The maximum capacity of the autosampler tray is 30 desorption tubes. The desorption tubes (length, 10 cm; i.d., 3 mm) were partly (*ca.* 30 %) filled with Tenax TA (80-100 mesh). A glass-wool plug was firmly pushed on the top of the Tenax material and was fixed with a metal clamp. The tubes were preconditioned by heating under a stream of helium at 280 °C for at least 4 h. The sample (up to 500 µl), dissolved in ethyl acetate, was brought onto the Tenax material in portions of 100 µl, using a 100-µl syringe. After each portion, the solvent was partially removed by leading a nitrogen flow of 80 ml/min through the tube for 1 min. After applying the last portion, the nitrogen flow was led through for 5 min. The tubes were placed in the autosampler tray. Residual ethyl acetate was removed just before desorption, by purging with helium for 999 s (when 500 µl sample was applied). Next, the sample was desorbed from Tenax by heating for 2 min at 210 °C. Meanwhile, the cold trap of the injection system (deactivated CPSil 8 CB; length, 1 m; i.d., 0.53 mm; film thickness, 5 µm) was kept at -60 °C with liquid nitrogen. Cooling was started before positioning of the tube which had to be desorbed. The temperatures of the TDAS valve and the interface between this valve and the desorption tube were kept at 140 °C. The analytes were reinjected from the cold trap via a temperature increase from -60 to 160 °C at 'infinite' rate. This flash heating signal started the program of the gas chromatograph. After desorption, the tube was reconditioned for 1 min at 210 °C in the TDAS, and subsequently as described above.

GLC configuration 5 (SPI-GC-PFPD)
This configuration consited of a Varian Star 3400 CX gas chromatograph (Palo Alto, CA, USA) equipped with a Septum Programmable Injector (SPI) and a pulsed-flame photometric detector

(PFPD). The analytical column was DB5-MS (Chrompack; length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm). Carrier gas (helium) pressure was set at 140 kPa.
The temperature program of the GC was as follows: 70 °C for 0.1 min, heating to 170 °C at a rate of 15 °C/min, 170 °C for 0 min, heating to 250 °C at a rate of 25 °C/min, 250 °C for 0 min, cooling to 70 °C at 'infinite' rate. The detector temperature was set at 200 °C. The detector was operated at the standard settings for sulfur, as specified by the manufacturer. The injected volume of sample was 3 μl.

GLC configuration 6 (TCT-GC-PFPD)
This configuration resembles configuration 5, albeit that the SPI was replaced with a TCT-injector (Chrompack). Up to 400 μl of sample in ethyl acetate was brought onto Tenax. The solvent was removed by leading a nitrogen flow of 80 ml/min through the Tenax. The Tenax tube was positioned in the TCT injector, after which the sample was desorbed at 210 °C for 6 min. Meanwhile, the cold trap was kept at -50 °C. The analytes were reinjected from the cold trap by flash heating to 170 °C.

II.3 Extraction of SM from blood

A known volume of blood was pipetted into a glass tube containing ethyl acetate to which a known concentration of D_8-SM was already added. This tube was kept in melting ice. Blood volumes of 0.1-2 ml were extracted with 1 ml of ethyl acetate, whereas 5-ml blood samples were extracted with 3 ml of ethyl acetate. The phases were mixed on a whirlmixer for 10 s, and then placed in an ultrasonic bath for 10 min. Next, the phases were separated by centrifugation. The ethyl acetate phases were transferred into glass vials and placed in the autosampler of either GLC configuration 1, 2 or 3, or were transferred onto Tenax for analysis with GLC configuration 4.

II.4 Extraction of SM from tissues

A weighed amount of tissue was transferred into a glass tube. After adding an approximately equal volume of cold ethyl acetate, containing a known concentration of D_8-SM, the tissue was homogenized using a Polytron ultra-thurrax. After 10 min of ultrasonication the phases were separated by centrifugation at 20,000 rpm for 10 min. The ethyl acetate phase was removed and stored at –70 °C until analysis.

II.5 Calibration curves

Calibration curves were constructed relating the peak height of SM to that of the internal standard, D_8-SM. Separate curves were constructed for the SM concentration range from near the detection limit (*ca.* 10 pg/ml) up to 100 ng/ml, and for the range from 100 ng/ml up to 5 μg/ml.

II.6 Vapor exposure of animals

Apparatus for generation of sulfur mustard vapor
The apparatus for controlled generation of SM vapor in air was adapted from that used for generation of nerve agent vapor (Benschop and Van Helden 1993). Due to the lower volatility of SM in comparison with soman and sarin, the generation apparatus had to be thermostatted at 35-40 °C in order to generate sufficiently high SM vapor concentrations.

Apparatus for nose-only exposure of hairless guinea pigs to sulfur mustard vapor
The exposure apparatus was also adapted from that used for exposure to nerve agents (Benschop and Van Helden 1993). The internal volume was reduced and the pathways were shortened. Furthermore, the teflon front chamber from which the animal breathes was replaced with a stainless steel front chamber. The apparatus is described in detail in Chapter III, paragraph 6.

Apparatus for percutaneous exposure of hairless guinea pigs to sulfur mustard vapor
A chamber was designed and built for percutaneous exposure of hairless guinea pigs to SM vapor, made of glass and stainless steel. The anesthetized animal was immobilized on a grid, with a fixation fork placed around its neck. The animal breathed clean air via an opening in the front panel of the apparatus, which was sealed around the nose of the animal with a rubber mask. A carotid cannula protruded through a silicone rubber septum in the front panel. A sampling point connected to a gas chromatograph was present, enabling semi-continuous monitoring of the SM vapor concentration. Furthermore, provisions for temperature control inside the chamber were present. The exposure chamber was connected to the vapor generation apparatus. The ensemble was place inside a perspex cabinet with air suction.
A detailed description of the apparatus is presented in Chapter III, paragraph 10.

II.7 Isolation of DNA from various tissues

The following protocol concerns the processing of 1 g of liver tissue.
Frozen liver was homogenized with a Potter-homogenizer in 0.25 M sucrose, 0.1 M EDTA, pH 7.4. Nuclei were isolated by centrifugation for 10 min at 3,000 rpm. The nuclear pellet was washed with the abovementioned buffer and was resuspended in 5 ml sucrose, 25 mM EDTA, 1 % Triton, pH 7.4, and incubated for 40 min at 4 °C. The chromatin was isolated by centrifugation for 10 min at 3,000 rpm and washed three times with 10 mM Tris.HCl, pH 7.4. Next, the pellet was resuspended in 2.5 ml TEN buffer (20 mM Tris.HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl).
The protein components of the chromatin were degraded by addition of 2.5 ml TEN-buffer, containing 1 % (w/v) sodium dodecyl sulfate (SDS) and 100 µg Proteinase K/ml, and overnight incubation at 37 °C. Next, protein was removed by three successive extractions with 5 ml of phenol (saturated with Tris.HCl, pH 8.0), phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), and chloroform/isoamyl alcohol (24:1, v/v), respectively. The nucleic acids in the aqueous phase were precipitated by addition of 2.5 volumes of cold absolute ethanol (-20 °C). The precipitate was washed twice with 70 % ethanol, dried *in vacuo*, and finally dissolved in 5 ml of 10 mM Tris.HCl, pH 7.4, 1 mM EDTA (TE-buffer).
RNA was degraded by addition of RNAse A (75 µg/ml) and RNAse T1 (75 U/ml) and incubation at 37 °C for 2 h. After this incubation, 500 µl of a 10 times more concentrated TEN-buffer and 500 µl of 10 % SDS were added. Next, the same series of extractions as described above was repeated in order to remove enzymes and degraded RNA. The purified DNA was precipitated, washed with 70 % ethanol, dried, and dissolved in 1 ml TE-buffer.
The DNA concentration was determined from the absorbance at 260 nm, on the assumption that the A_{260} of a 1 mg/ml solution of native DNA is 20. The overall purity of DNA was verified by determination of the A_{260}/A_{280} and the A_{260}/A_{230} ratios of the DNA solution.
Isolation of DNA from other organs and epithelial tissues was carried out according to the procedure described above, except that the separate isolation of cell nuclei was omitted. After homogenization of the tissue, the isolation procedure is started with Proteinase K and SDS treatments (*vide supra*).
The yield of DNA was usually 1-1.5 mg per gram tissue.

Isolation of DNA by using a PureGene DNA isolation kit (Biozym)
In later experiments DNA was isolated by using a commercial preparation, i.e., the PureGene DNA isolation kit (Biozym, Landgraaf, The Netherlands). This enabled a much faster DNA-isolation procedure which could also be applied to our tissue samples without loss or destruction of DNA-adducts. The procedure was as follows:
Fresh or frozen tissue (10-20 mg) was added to 600 µl of cell lysis solution at 0 °C. A homogeneous suspension was prepared with a mini-Potter-homogenizer. Six µl of a proteinase K solution (10 mg/ml) was added, and the sample rotated at 37 °C overnight, until the tissue was dissolved. Next, 2 µl of RNase A solution (10 mg/ml) was added and the sample was incubated at 37 °C for 1 h. Subsequently, 200 µl of protein precipitation solution was added, stirred for 20 s, and centrifuged for 3 min at 14,000 rpm. The supernatant was transferred into a 2-ml Eppendorf tube containing 600 µl of 2-propanol. Mixing was performed until the DNA was completely precipitated, followed by centrifugation (1 min, 14,000 rpm). The supernatant was discarded and 600 µl of 70% ethanol was added. The DNA was thoroughly washed by stirring for a short time period, followed by centrifugation (1 min, 14,000 rpm). Ethanol was carefully discarded. The DNA pellet was dried in a 'Speedvac' or in a 37 °C-incubator for about 1 h. Next, the DNA was dissolved in 0.1TE-buffer (1 mM Tris.HCl, 0.1 mM EDTA, pH 7.4).
The overall purity of the DNA was verified by determination of the A_{260}/A_{280} and the A_{260}/A_{230} ratios of the DNA solution. The yield of DNA was usually 25-100 µg per 10 mg tissue.
In the case of DNA isolation from blood, RBC Lysis Solution (900 µl) was first added to blood (300 µl) in order to lyse the red blood cells and the mixture was centrifuged at 14,000 rpm for 20 s. The pelleted white blood cells were lysed with Cell Lysis Solution (300 µl) and further processed as described above.

Isolation by using a DNA Isolation Kit for Mammalian Blood
Whole blood (300 µl) was added to Red Blood Cell Lysis Buffer (900 µ1) of the DNA Isolation Kit for mammalian blood (Boehringer, Mannheim, Germany). After gently shaking for 10 min, the mixture was centrifuged at 14,000g for 20 s. The supernatant was discarded and the pellet was resuspended in the residual supernatant. White Cell Lysis Buffer (300 µl) was added and mixed thoroughly by vortexing. After a clear solution was obtained (45 min at 37 °C), RNAse A (1.5 µl; 50 µg/ml) was added to a final concentration of 0.02 µg/ml and incubated at 37 °C for 15 min. After cooling to room temperature, Protein Precipitation Solution (150 µl) was added. The mixture was vortexed thoroughly and then centrifuged at 12,000g for 10 min. The supernatant was poured carefully into a new tube and 2 volumes ethanol were added at room temperature, gently mixed and centrifuged at 12,000g for 10 min. The pellet was washed with 70% ethanol (1 ml), dried at 37 °C for 15 min and solved overnight under continuous vibration at room temperature in TE buffer (100 µl).

DNA isolation from hairless guinea pig skin
Skin biopts (about 1 cm^2) were incubated overnight at 4 °C in a 3-cm diameter Petri-dish in a solution of dispase (2.4 mg/ml in PBS; 3 ml) in order to separate the epidermis from the dermis. The epidermis was transferred into an Eppendorf tube, after which the lysis solution (600 µl) of the PureGene kit (Biozym) was added. The sample was further processed as described above.

DNA denaturation
Double-stranded calf thymus DNA or DNA from SM exposed tissues were made single-stranded by thermal denaturation in TE buffer containing 4.1% formamide and 0.1% formaldehyde (50 µg DNA/ml) at 52 °C for 15 min, followed by rapid cooling on ice and storage at -20 °C. In later experiments thermal denaturation was carried out in 0.1TE buffer with the same amounts of formamide and formaldehyde.

II.8 Immuno-slot-blot assay of sulfur mustard adducts to DNA

In the immunoslotblot assay (ISB, Nehls *et al.*, 1984) as originally applied, the thermally denaturated DNA solution of 50 µg/ml, was 10-fold diluted in PBS (0.14 M sodium chloride, 2.6 mM potassium chloride, 8.1 mM disodium hydrogen phosphate and 15 mM potassium dihydrogen phosphate, pH 7.4). Next, one hundred µl of this DNA solution were spotted on a nitrocellulose filter (pore size 0.1 mm; Schleicher and Schuell). After washing with PBS, the DNA was adsorbed to the filter by drying for 15 min at 37 °C. Next, the filter was treated with 5 % milk powder (Campina, Eindhoven, The Netherlands; less than 1% fat) in PBS containing 0.1 % Tween 20, under continuous shaking at room temperature for 30 min in order to prevent nonspecific antibody binding. After washing, the filters were treated and incubated with N7-SM-gua monoadduct-specific monoclonal (2F8) antibody (1:500 dilution of culture supernatant in PBS containing 0.1 % Tween 20 and 0.5 % milkpowder) at 4 °C overnight. After washing with PBS containing 0.1 % Tween 20, the filters were incubated with conjugated second antibody (rabbit-anti-mouse-Ig-horse radish peroxidase, diluted 1:1000 in PBS containing 0.1 % Tween 20 and 0.5 % milkpowder). The filters were incubated for 2 h at room temperature. After a final washing step, peroxidase activity was revealed with a Boehringer enhanced chemiluminescence blotting detection system. Peroxidase labeled antibodies catalyze the oxidation of luminol resulting in emission of light in the presence of H_2O_2 (two solutions supplied by the manufacturer, added in a ratio of 100:1). After 1 min the filters were transferred to cassettes containing photographic film (Hyperfilm ECL) and exposed after 5 min to the filters for 10 -120 s. The signal was quantified by scanning the developed film with an Ultroscan XL densitometer (Pharmacia, Uppsala, Sweden).
The lower detection limit of the ISB-assay was reached when 0.5 µg DNA was spotted on the filter (DNA exposed in its double-stranded form in solution for 1 h to 1 nM SM at 37 °C, and subsequently made single-stranded according to the abovementioned procedure). The adduct level at the detection limit is 1 modified guanine amongst $2x10^7$ nucleotides (based on calibration with HPLC with electrochemical detection (Benschop and Van der Schans, 1995). This corresponds with 0.08 fmol of adduct per spot of 0.5 µg DNA. Since 1 g tissue yields 1-1.5 mg DNA (with the phenol extraction method), the lower detection limit can also be expressed as 0.1-0.15 pmol adduct/g tissue.

Optimized immuno-slot-blot procedure for sulfur mustard adducts to DNA

In the course of this project, several aspects of the ISB-procedure were modified. The procedure described below is currently the most optimal. In the immunoslotblot assay (ISB) the single-stranded DNA containing 7-SM-gua was first slotblotted onto a nitrocellulose filter. Thermal denaturated DNA was diluted in PBS to a final concentration of 5 µg/ml. The solution (200 µl) was spotted onto a nitrocellulose filter. Ten positions on the 96-blots filter were occupied by calibration samples of DNA with adduct levels in the range of 0-10 7-SM-gua/10^7 nucleotides. All samples were blotted in duplicate on the same filter. After blotting, the slots were rinsed with PBS. The filters were dried at the air and the DNA was immobilized by UV-crosslinking (50 mJ/cm^2). The next steps in the procedure, treatment with blocking solution, 1st antibody (2F8, directed against 7-SM-gua in DNA) and 2nd antibody (rabbit-anti-mouse-Ig-horse radish peroxidase), were the same as described in the previous paragraph. Next, the solutions A and B of the chemiluminescence blotting detection system were mixed (100:1) and equilibrated for 1 h at 25 °C before addition to the filter. The filters were incubated for 1 min in substrate and then placed in a plastic bag. Excess liquid was removed by applying pressure onto the filters. Next, the filters were placed in a luminometer and the chemiluminescence was measured.
The lower detection limit of this modified ISB-assay was reached when 1 µg DNA was spotted on the filter (DNA exposed in its double-stranded form in solution for 1 h to 0.05 nM SM at 37 °C, and subsequently made single-stranded according to the abovementioned procedure). The adduct level at the detection limit was 1 modified guanine amongst $4x10^8$ nucleotides (based on calibration with HPLC with electrochemical detection (Benschop and Van der Schans, 1995). This corresponds with 0.008 fmol of adduct per spot of 1 µg DNA. Since 10 mg tissue yielded

25-100 µg DNA (with the PureGene kit), the lower detection limit can also be expressed as 0.02-0.08 pmol adduct/g tissue.

II.9 Immunofluorescence assay of sulfur mustard aducts to DNA

Preparation of skin cryostat sections
After exposure to SM, a piece of the skin (*ca.* 1 cm^2)was cut from the central part of the treated area and fixated in methanol/acetic acid (3/1 v/v, 1.5 h at 4 °C), rehydrated overnight in 70% ethanol at 4 °C, followed by incubation in 5% sucrose at 4 °C for 1.5 h. Next, the pieces were stretched between microscope slides and stored at -20 °C.
For the preparation of cryostat sections a small piece of skin was embedded in Tissue Tek (O.C.T. compound, Miles Inc., Elkhart, USA). Subsequently, cryostat sections (5 µm thickness) were prepared at -35 °C with a cryostat microtome (2800 Frigocut, Rechert-Jung, Leica, Rijswijk, The Netherlands) on slides precoated with a solution (2%) of 3-aminopropyl tri-ethoxysilane in acetone. The slides were stored at room temperature.

Immunofluorescence microscopy
Quantitative immunofluorescence microscopy to analyse the formation of sulfur mustard adducts to DNA was performed on the skin cryostat sections. Several variations in the processing of the skin cryostat sections were tested to improve the sensitivity. It appeared that after fixation on aminoalkylsilane-precoated slides, the following procedure provides to the best results:
- 30 min hydratation at room temperature;
- treatment with RNase (100 µg/ml) at 37 °C for 1 h;
- denaturation of the DNA with 70% formamide in 0.14 M NaCl containing 0.01 M sodium citrate at 70 °C for 5-10 min, followed by treatment with 3% formaldehyde (1 min), 70% ethanol (1 min) and washing with 50% ethanol;
- treatment with proteinase K (2 µg/ml, 10 min at 37 °C);
- precoating with TBS (20 mM tris-HCl, 150 mM NaCl, pH 7.4) + 5% milkpowder (30 min at room temperature);
- treatment with antibody (specific for SM-modified DNA, 2F8, in TBS containing 0.05% Tween 20 and 0.5% gelatin, overnight at 4 °C);
- treatment with a second antibody, FITC-labeled 'goat-anti-mouse' (GAM-FITC, Southern Biotechnology Associates, Birmingham, AL), 100-fold diluted in TBS containing 0.05% Tween 20 and 0.5% gelatin, (2 h at 37 °C);
- counterstaining with propidium iodide (100 ng/ml, 10 min at room temperature).

Twin images were obtained with a laser scanning microscope (LSM-41, Zeiss, Oberkochen, Germany). The fluorescence of the FITC group and of the propidium iodide were measured consecutively to visualize the DNA in the nuclei. The first one, the fluorescein staining, was used to determine the ss-DNA content. Images were digitized in a format of 512x512 pixels, the brightness of which ranges from 0 to 255 arbitrary units. The second image of the same nuclei, the propidium iodide staining, served to localize nuclei on the image. Recognition of nuclei and calculation of the fluorescein-fluorescence therein was performed with the image-processing software package SCIL-Image (CBP, Delft, The Netherlands) on a remote disk of a workstation (Silicon Graphics 4D/35). On this machine, image processing, using SCIL-image as a basic toolbox, was done automatically (in batch) or in interactive way.

Alternatively, fluorescence measurements were performed using the CCD system. This consisted of a liquid-nitrogen cooled CCD camera (LN_2 Astromed Ltd., Cambridge, England) placed on top of a Leitz Orthoplan fluorescence microscope. The specimen was excited by light of a 100 watt DC mercury-arc lamp, filtered with a bandpass filter, adapted to the fluorochrome used (for FITC: BP 485/20). The dichroic mirror used was DM 510. The lamp illuminated the total specimen, resulting in a fluorescence image selected by means of the emission filter BP

515-560. This image was projected onto the CCD chip of the camera using a 40x oil-immersion objective. The camera was controlled by special image pre-processing hardware (Astromed Ltd), placed in a personal computer (Unix). A custom-written recording program was run on this PC, essentially similar to the one in the LSM set-up, that allowed recording of large sequences of images. These were directly transported via the local area network to the same disk of the workstation as in the case of the LSM set-up. The user chosen type of image processing could be performed in the same way as with the LSM.

II.9 Animal experiments

Animals
Male hairless guinea pigs [400-500 g; species identification Crl:IAF(HA)BR] were purchased from Charles River Wiga GmbH (Sulzfeld, Germany). The animals were allowed to eat and drink *ad libitum*. They were allowed to acclimatize to their new environment for at least 1 week before they were used in any experiment. The protocols for the animal experiments were approved by the TNO Committee on Animal Care and Use.

Acute toxicity of SM
The 96-h i.v. LD50 of SM was determined by administration of 5 different doses of SM to groups of 6 hairless guinea pigs via the jugular vein.
The 96-h LCt50 for 5-min nose-only exposure to SM vapor was determined by exposure of groups of 8 hairless guinea pigs to five different Ct-values.
Survival times were assessed by housing the animals individually in a cage equipped with an ultrasonic device which detects respiratory movements. Twice a day during 4 days after SM administration, the animals were treated with the long-lasting analgesic buprenorfine (Temgesic®, 0.02 mg/kg s.c.). The animals had access to food and water *ad libitum*.
Mortality rates were scored after 96 h, after which the LD50 and LCt50 values were established via probit analysis (Litchfield and Wilcoxon, 1949).

Toxicokinetic studies
Hairless guinea pigs were anesthetized with racemic ketamine hydrochloride (Vetalar®, 80 mg/kg i.m.). A carotid cannula was installed, through which blood samples were drawn. For experiments involving i.v. administration of SM, the jugular vein was made accessible. In the inhalation experiments, the animals were fixated in modified Battelle tubes as described elsewhere[8]. For the percutaneous experiments, the animals were fixated on the grid of the exposure chamber.
Just before administration of, or exposure to SM, a blood sample was taken via the carotid artery, after which a corresponding volume of saline was administered via the same cannula. Throughout the toxicokinetic experiments, blood samples were taken at various time-points. After taking the final blood sample the animals were sacrificed with an overdose of Nembutal®, and various tissues were sampled for analysis of SM and 7-SM-gua.

Protective efficacy of scavengers
Five groups of 6 hairless guinea pigs were pretreated (i.p.) with 5 mmoles/kg of N-acetyl cysteine (NAC) or cysteine isopropylester (CIPE), 1 min prior to a 5-min nose-only exposure to various concentrations of SM vapor in air. At 96 h the mortality rates were scored, from which the LCt50 values in scavenger protected animals were calculated via probit analysis (Litchfield and Wilcoxon, 1949).

Protective efficacy of topical skin protectants
On the backs of groups of 6 anesthetized, immobilized animals 12 circular spots of *ca.* 1 cm^2 were marked with ink. On 4 of these spots topical skin protectant (TSP) 1511 was applied, *ca.*

30 mg on each spot. On four other spots TSP 2701 was applied, also *ca.* 30 mg per spot. Spreading the TSP's resulted in a layer thickness of approximately 0.25 mm. The four remaining spots remained unprotected and served as positive controls in the SM challenges. The control, TSP 1511 and TSP 2701 positions shifted two spots for each subsequent animal, which means that within the group of 6 animals each of the twelve spots was twice a control, twice covered with TSP 1511 and twice covered with TSP 2701.
In order to establish the protection against a challenge with liquid SM, a 1-µl droplet of neat SM was applied on each of the spots. After 2 h, the exposed sites were decontaminated by means of tissue paper. Simultaneously, the TSP's were also removed. Two h after decontamination, skin biopts were taken for determination of the DNA adduct concentration in the epidermis.
In order to establish the protection afforded by TSP's against a challenge with SM vapor, the animals were exposed to a Ct of 1,500 mg.min.m^{-3} in 10 min. At 2 h after ending the exposure, skin biopts were taken for determination of the DNA adduct concentration in the epidermis. This exposure level was chosen on the basis of the results of experiments in which groups of 6 hairless guinea pigs were exposed to Ct's of 100, 500, 1,500 and 3,000 mg.min.m^{-3}.

II.10 Curve-fitting of toxicokinetic data

Curve-fitting of the measured concentration-time courses was performed by nonlinear regression with the BMDP-3R program (University of California, Los Angeles, CA, USA) on a personal computer equipped with an Intel P-5 processor, as decribed previously (Benschop and De Jong, 1990).
The data were fitted to a bi- and a triexponential equation :

$$[SM]_t = A*e^{-\alpha t} + B*e^{-\beta t} \qquad \text{(eq. 1)}$$

$$[SM]_t = A*e^{-\alpha t} + B*e^{-\beta t} + C*e^{-\gamma t} \qquad \text{(eq. 2)}$$

by calculation of the parameters A, B, C, α, β, and γ. In these equations, $[SM]_t$ is the blood concentration of SM at time t. The F-ratio test (Boxenbaum *et al.* 1974) was used to determine whether the data were significantly better described with a triexponential equation than with a biexponential equation.
Several toxicokinetic parameters were calculated:

the area under the curve (AUC) $AUC = A/\alpha + B/\beta$ (eq. 3)

the total body clearance (Cl) $Cl = Dose/AUC$ (eq. 4)

the rate constant of transport from compartment 2 to compartment 1 ($k_{2,1}$)

$$k_{2,1} = (A*\beta + B*\alpha)/(A + B) \qquad \text{(eq. 5)}$$

the rate constant of total body clearance (k_{el})

$$k_{el} = \alpha*\beta/k_{2,1} \qquad \text{(eq. 6)}$$

the rate constant of transport from compartment 1 to compartment 2 ($k_{1,2}$)

$$k_{1,2} = \alpha + \beta - k_{2,1} - k_{el} \qquad \text{(eq. 7)}$$

the blood concentration at time 0 (C_0) $C_0 = A + B$ (eq. 8)

the half-life of distribution ($t_{½,dis}$) $t_{½,dis} = \ln 2/\alpha$ (eq. 9)

the terminal half-life ($t_{½,el}$) $t_{½,el} = \ln 2/\beta$ (eq. 10)

the volume of the central compartment (V_1)

$V_1 = Dose/C_0$ (eq. 11)

the distribution volume at steady state (V_{dss})

$V_{dss} = V_1(1 + k_{1,2}/k_{2,1})$ (eq. 12)

the mean residence time (MRT) $MRT = (A*(1/\alpha)^2 + B*(1/\beta)^2)/AUC$ (eq. 13)

III. RESULTS AND DISCUSSION

III.1 GAS CHROMATOGRAPHIC ANALYSIS OF SULFUR MUSTARD IN BIOLOGICAL MATERIALS

III.1.a. Extraction of sulfur mustard from blood and tissue samples

Based on our experience with the gas chromatographic trace analysis of nerve agents in biological materials and on the preliminary reports of Maisonneuve *et al.* (1993) on the gas chromatographic analysis of SM in rat blood, it was checked whether SM could be extracted with ethyl acetate from guinea pig blood samples stabilized with 18 % aqueous sodium chloride solution. Extraction recoveries from blood should be as high as *ca.* 90 %, whereas the limit of detection with gas chromatography with FID should be *ca.* 3 ng/ml blood (Maisonneuve *et al.* 1993).

Once these results were reproduced, the extraction procedure needed to be optimized with respect to several aspects. First of all, the stabilization procedure for SM in blood is essential to obtain valid data. Due to the high reactivity of SM towards the biological matrix this aspect required special attention. The influence of parameters such as pH, temperature, concentration of chloride ions and incubation time were studied. Furthermore, the extraction of SM from various tissues, i.e., fat, bone marrow, spleen, liver, lung and dorsal skin was studied using the same procedure as for blood, for which some adjustments to the procedure might be necessary. Since the reported recovery from liquid-liquid extraction is high, no significant increase in sensitivity could be gained by further optimizing this step in the analysis. It remained worthwhile, however, to study whether solid-liquid extraction with C_{18}- or other cartridges would be more convenient, with respect to e.g. removal of contaminants from the samples or time consumption.

The extraction of sulfur mustard (SM) was studied using gas chromatographic configuration 1 (GC-FID). On this configuration SM and deuterated SM (D_8-SM), which was used as the internal standard in the extraction procedure, were completely resolved. The absolute detection limit for SM appeared to be *ca.* 400 pg. The calibration curve of SM appeared to be linear up to a concentration of 3.5 µg/ml. The within-day variability for this system, based on SM peak height, appeared to be 4.8 %, the between-day variability was 11 %.

A long-term study of the stability of SM and D_8-SM in ethyl acetate was started in January 1995. Solutions in ethyl acetate containing respectively 171 ng D_8-SM and 250 ng SM per ml, 16 µg D_8-SM and 25 µg SM per ml, and 34.2 ng D_8-SM/ml were prepared. One portion of each of these solutions was kept at room temperature, another portion at -20 °C. Once every two weeks the concentration of each of the solutions was determined with GLC configuration 1. Some variation in the concentration was observed with time, but there are no indications for degradation of SM or D_8-SM in any of the solutions. However, in a solution of SM in saline of 100 µg/ml, SM appeared to degrade at room temperature (*ca.* 22 °C) with a half-life of 26 min. In melting ice (*ca.* 4 °C), degradation proceeds *ca.* ten times more slowly. Therefore, it was decided to perform extractions from aqueous solutions such as saline and blood at 4 °C.

SM and D_8-SM (*ca.* 250 µg/ml) were spiked into saline and extracted with cold (4 °C) ethyl acetate in 1:1 volume ratio. The phases were mixed on a whirlmixer for 10 s and then placed in an ultrasonic bath for 10 min. Next, the phases were separated by centrifugation. Ultrasonication appeared to improve the extraction recovery. With a 1-min ultrasonication the absolute recovery of SM was *ca.* 60 %, which increased up to 91 % for a 10-min ultrasonication. Extending the duration of the ultrasonication beyond the 10-min period did not improve the extraction recovery. In fact, the temperature of the sample increases with the duration of ultrasonication, which may accelerate degradation of SM in the sample.

The extraction recovery was not significantly improved by increasing the saline:ethyl acetate phase ratio from 1:1 to 1:5. However, decreasing the saline:ethyl acetate ratio down to 5:1 had a detrimental effect on the recovery. The lowest phase ratio which can be used for an acceptable

recovery is 3:2. Under all extraction conditions, the recovery relative to D_8-SM was 99 %, which indicates that D_8-SM is indeed a suitable internal standard for SM.
Solid phase extraction of SM from saline by elution with ethyl acetate from a SepPak C_{18} cartridge appeared to lead to a recovery of *ca.* 70 %. Of the recovered amount of SM, 97 % was eluted in the first ml of ethyl acetate. Again, the recovery relative to D_8-SM was near 100 %.
Extraction of SM from guinea pig blood was performed by mixing on a whirlmixer followed by ultrasonication and centrifugation as described above. The absolute recovery of SM was 86 ± 4 % (n=6) at the 500-ng SM/ml blood level, and 91 ± 4 % (n=6) at the 1-ng/ml level, whereas the recovery relative to D_8-SM was 99 ± 3 %. Validation of the analytical procedure in blood samples was performed in the concentration range from *ca.* 10 pg/ml up to 5 μg/ml, using analysis by TDAS-GC-MS (GLC configuration 4, *cf.* Section II.2). Results are presented in Table 1.

Table 1. Results of the validation of the procedure for analysis of SM in hairless guinea pig blood. Recoveries are relative to the internal standard.

SM added to blood (ng/ml)	SM measured in blood (ng/ml, ± s.d., n=6)
5000	5005 ± 100
500	495 ± 15
50	51 ± 2
1	0.99 ± 0.03
0.4	0.39 ± 0.04
0.01	Failed

Table 1 shows that the validation at a concentration of SM in blood at 10 pg/ml has failed. The peak of SM could be detected with the MS, but quantification was tedious and associated with a high standard deviation of *ca.* 20 %. Validation at the level of 400 pg/ml was more successful, albeit that the standard deviation was *ca.* 9 %. The absolute recovery of SM at this concentration level was still as high as 89 %. At higher concentrations of SM added to blood, the standard deviation of the analysis was *ca.* 2-3 %, whereas the recovery relative to D_8-SM was around 100 %.
During the toxicokinetic studies the concentration of SM in blood was in by far most of the samples well above the level of 400 pg/ml. Therefore, we accepted this validation as being adequate for the purpose of the current study, albeit not to our full satisfaction.

SepPak C_{18} solid phase extraction the recovery of SM was in the range of 50-60 %. From the appearance of the chromatograms it was concluded that solid phase extraction does not provide a higher selectivity than liquid-liquid extraction. Furthermore, the solid phase extraction procedure is more labor intensive, and we therefore preferred to continue with the liquid-liquid extraction procedure.
We performed the extraction in the actual toxicokinetic experiments by pipetting the blood sample into a tube containing ethyl acetate to which D_8-SM is already added. We verified that the final concentration of D_8-SM in the ethyl acetate phase after this procedure was the same as when D_8-SM was first added to the blood sample and subsequently extracted.
After ultrasonication the samples do not need to be centrifuged immediately. When the samples were kept in melting ice, no degradation of SM and/or D_8-SM was observed up to 1 h. This is a useful finding, since it will be more convenient to gather several samples for centrifugation when performing a toxicokinetic experiment. Usually, the sonicated samples were centrifuged within 30 min.
Under these extraction conditions the concentration of chloride ions, in the range of 0.17 up to 3.4 M, did not significantly influence the recovery of SM, which was spiked at a concentration of 156 ng/ml blood. This seems to be in contradiction with findings of Maisonneuve *et al.* (1993) and others, but it has to be realized that our method is based on immediate extraction of

the blood sample. During the performance of the toxicokinetic experiments the samples were always extracted immediately after they were drawn from the animal.
The influence of the pH on the extraction recovery from blood was studied with a concentration of 156 ng SM/ml blood in the pH range of 5 to 7.5. In this pH range the absolute extraction recovery remained unaltered at 92 ± 7 %, whereas at lower or higher pH the recovery appeared to decrease, most likely due to degradation of SM. Since the physiological pH is *ca.* 7.4 no problems with respect to the stability were anticipated with our extraction method.
After addition to homogenates of lung, liver, fat, spleen and bone marrow at a concentration of *ca.* 0.5 µg/g homogenate, SM was extracted with recoveries (± s.d., n=3) of 87 ± 5, 95 ± 4, 101 ± 4, 98 ± 4, and 98 ± 9 %, respectively. These recoveries are comparable to the value found for extraction from blood. Obviously the tissues containing SM have to be homogenized prior to extraction in the actual experiments, which will take some time since they cannot all be processed at the same time. Depending on how long processing will take, loss of SM due to hydrolysis or binding in the tissue can be expected. Freeze-drying of the tissue samples appeared to be unsuitable, as this procedure leads to a considerable loss of SM. The most promising approach seemed to be to freeze the tissue samples in liquid nitrogen until processing, which we performed by homogenizing the tissue sample directly with ethyl acetate by means of an Ultrathurrax.
Furthermore, we confirmed that SM was not formed from thiodiglycol under the extraction conditions used.

III.1.b. Comparison of gas chromatographic detectors for sulfur mustard

Only a few papers have been published concerning the analysis of intact SM in biological matrices. In nearly all reported methods, gas chromatography is used to analyze SM. This is a logical choice in view of the volatility of SM, the versatility of gas chromatography in general, and because of the combination of selectivity with superior detection limits, especially in the case of two-dimensional chromatography. The reported detection systems are flame-ionization detection (FID; Maisonneuve *et al.*, 1993), flame photometric detection (FPD; Zhang and Wu, 1987), electron capture detection (ECD; Heyndrickx *et al.*, 1984) and mass-spectrometric detection (MS; Vycudilik, 1985; Vycudilik, 1987, Drasch *et al.*, 1987).
For detection of sulfur containing compounds, a flame photometric detector (FPD) is mostly first choice, as it combines ease of operation with a high selectivity and a detection limit of ca $5*10^{-12}$ g S/s, corresponding with a detection limit of *ca.* 45 pg of SM at a peak width at half the peak height of 2 s (Degenhardt, 1992). With this configuration, in combination with the high extraction efficiencies, we anticipated a detection limit of 45 pg SM/ml blood or even somewhat better. However, preliminary experiments with electron capture detection (ECD) suggested that a detection limit of 5 pg SM/ml blood or g tissue could be obtained with this detector (Degenhardt, 1992). ECD tends to be less stable and reliable than FPD in routine analysis of biological samples due to contamination by matrix components, which is an important drawback of ECD in comparison with FPD. However, we envisaged that this problem could be circumvented by using ECD only in combination with two-dimensional chromatography, since in such a configuration the fraction of the sample that is injected onto the analytical column and actually reaches the detector is practically devoid of contaminants.
The limit of detection of SM with ECD appeared to be *ca.* 10 pg, which was somewhat disappointing. A calibration curve in the range of 10 ng/ml up to 150 µg/ml could be described with a second order polynoma. Increasing the reference current of the ECD from 1 to 5 mA in steps of 1 mA resulted in an increase of the signal offset which reduced the sensitivity of detection. Therefore, the reference current was set at 1 mA. The same effect was observed upon lowering the pulse voltage from 5 V to 1 V. Increasing the nitrogen flow of the ECD reduced the offset, but did not improve the sensitivity.
The sensitivity of ECD was compared with sulfur chemiluminescence detection (SCD), FPD and mass spectrometric detection (MS).

The comparison between ECD and SCD was performed at the Technical University of Eindhoven on a straightforward GLC configuration with a CPSil 5 column. A solution of SM in hexane was injected into the system. The SCD was a Sievers Model 350 B. On the basis of the manufacturer's specification a detection limit of 15 pg of sulfur was calculated, which corresponds with *ca.* 75 pg of SM. The actually measured detection limit appeared to be 78 pg of SM (S/N=3), which is almost equal to the specified value. The detection limit with ECD was 17 pg under comparable conditions. Therefore, SCD is not an suitable alternative for ECD in terms of sensitivity. However, SCD appeared to offer a better selectivity than ECD. Obviously, since only standard solutions of SM were injected, the difference in selectivity towards biological samples is unknown.
FPD appeared to be more selective but at least one order of magnitude less sensitive than ECD. Therefore, FPD is nót an attractive substitute for ECD. Recently, the pulsed flame photometric detector (PFPD) has been introduced (Amirav and Jing, 1995). The detection limit for sulfur with this new detector is reported to be 180 fg/s, which can even be improved to 30 fg/s with a sulfur doping method. The PFPD is reported to be more selective than FPD. Furthermore, the linear dynamic range of the PFPD is increased up to one order of magnitude in comparison with FPD. The PFPD is now commercially available from Varian. We had the opportunity to test this detector at Varian in Paris. Even with a non-optimized system, the detection limit for SM was at least one order of magnitude lower than that obtained with the FPD. This means that the PFPD is as sensitive as the ECD, but has the high selectivity of FPD. Since the PFPD cannot be placed onto a Carlo Erba GC it appeared necessary to buy a complete Varian system. Fortunately, we were able to find funding within our laboratory to purchase a Varian GC with on-column injector and PFPD.
Meanwhile, the detection limit of SM was studied with GC-MS. MS-detection was performed by positive electron impact ionization under semi-single ion monitoring conditions, at m/z 109 for SM and 115 for D_8-SM. The signal-to-noise ratio for an injected amount of SM of 3.9 pg of SM was 20, which corresponds with a detection limit of *ca.* 700 fg at S/N=3. Consequently, MS appears to provide for the most sensitive detection for SM of the detection principles that we studied. In addition, MS detection is highly selective, whereas the ability to identify the compound that is quantified can be considered as an important advantage.

III.1.c. Two-dimensional gas chromatography with large volume injection onto an on-column interface

Originally, we intended to use Thermal Cold Trap (TCT) injection as a means of large volume injection into the chromatographic system, since this proved to be a convenient and reliable technique in our previous studies on the toxicokinetics of soman and sarin (Benschop and Van Helden, 1993). In the course of those studies we purchased a TCT-autosampler which reduced the labor-intensity of the method in comparison with manual TCT-injection. Nevertheless, the extraction solvent had to be transferred onto the Tenax adsorbent. Furthermore, in the nerve agent studies a volume of *ca.* 5 ml of ethyl acetate had to be reduced to *ca.* 500 µl by evaporation, which was about the maximum volume that could be applied onto the Tenax. Therefore, the method remained rather time-consuming. We had tried to circumvent this problem at an earlier stage by using automated large volume on-column injection into the two-dimensional chromatographic system. Unfortunately, this technique appeared to be most unreliable and led to unreproducible results, and was abandoned in favor of TCT-injection (Benschop and Van Helden, 1993). The problems with the large volume on-column injection were the result of the high sensitivity of the injection to small variations in gas flow-rates and pressures in the system which were inevitable with that configuration. However, recently we have been able to solve these problems to such an extent that we were confident that this technique could be applied routinely for the purposes of the current study.
The newly developed configuration is shown in Figure 1. It comprises an HPLC autosampler, an HPLC pump and a gas chromatograph arranged for two-dimensional chromatography. Between

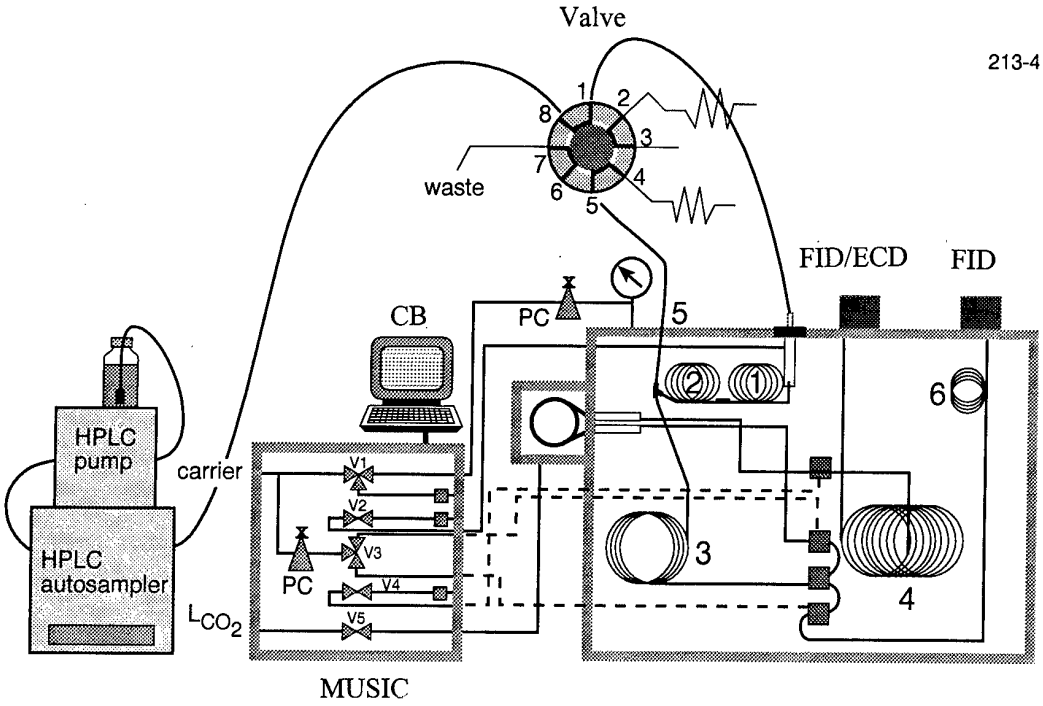

Figure 1. Schematic representation of the automated large volume GC-GC system (GLC configuration 2). (Valve), 8-port injection valve; (MUSIC), Multiple Switching Intelligent Controller; (PC), constant pressure valve; (V1-V5), valves; (CB), control box of the MUSIC; (1), retention gap; (2), retaining pre-column; (3), pre-column; (4), analytical column; (5), early solvent vapor exit; and (6), restriction capillary. FID and ECD were used as detectors. The FID connected to the restriction capillary served as a monitor detector.

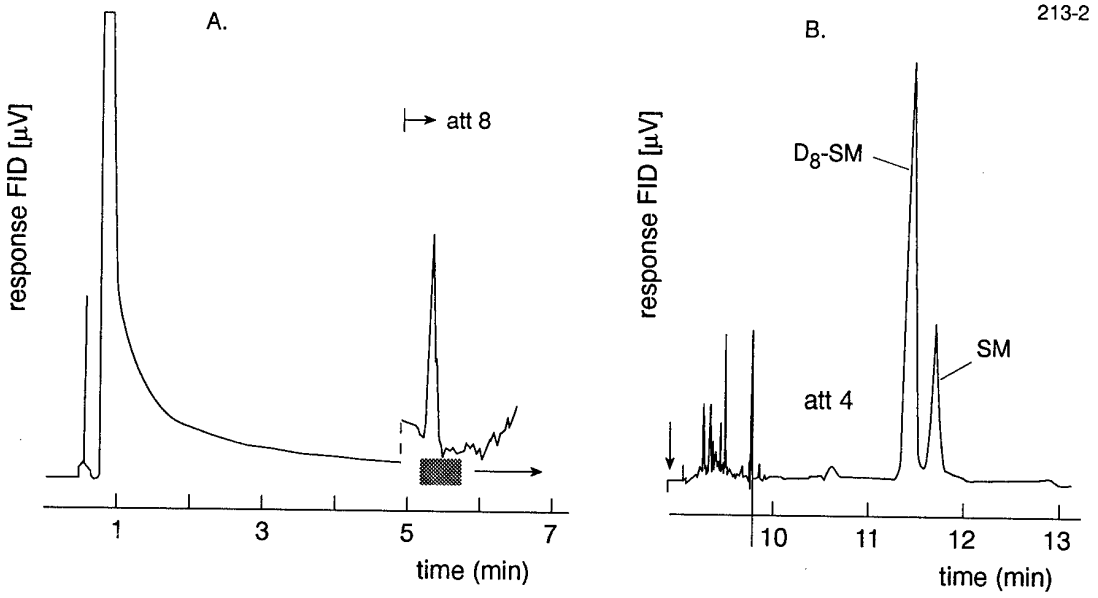

Figure 2. Pre-column (A) and analytical column (B) chromatograms after on-column injection of 1 µl of a standard solution of sulfur mustard (SM) and deuterated sulfur mustard (D_8-SM) in ethyl acetate (0.75 and 1.0 µg/ml, respectively) on GLC configuration 2.

the 'LC' and 'GC' parts of the configuration an 8-port valve is interconnected. The system is fully automated and is controlled by the autosampler and HPLC pump. At the moment of injection by the autosampler the time program of the HPLC pump is started, which in its term controls the position of the valve as well as the time program of the gas chromatograph. The flow from the HPLC pump transports the sample via the valve into the retention gap in the GC. This interface is set-up for Early Solvent Vapor Exit (ESVE), which is meant to vent the bulk of the solvent while the compounds of interest are being concentrated on a so-called retaining pre-column. Next, the analytes enter the precolumn of the two-dimensional system after which the fraction of interest is trapped and subsequently reinjected onto the analytical column. Initially, detection was performed with a flame ionization detector (FID). The various steps in the process were subsequently optimized.

Optimization of the MUSIC system

The original MUSIC system was equipped with a flow regulator for the injection onto the wide bore pre-column, and a pressure regulator for the analytical column. The interdependence of the carrier gasses of such a combined column system is evident. For our newly developed system we exchanged the flow regulator for a pressure regulator, which allows a rapidly responding carrier adjustment during injection. An additional valve had to be mounted just before this regulator so that the back-flush capabilities of the MUSIC system remained available. Next, a retention gap was connected in between the pre-column and the injector to prepare the system for large volume injection. The system was tested with on-column injection of a standard solution of SM and D_8-SM in ethyl acetate to study the retention behavior of the analytes on the combined pre-column combination as well as on the analytical column, see Figure 2. Optimum conditions were reached when the injection pressure (p_I) was set to 120 kPa and the pressure on the analytical column (p_A) to 78 kPa. The oven temperature was kept at 87 °C for 1 min, was subsequently increased up to 158 °C at a rate of 20 °C/min, and kept constant at this temperature for 15 min. The fraction eluting between 5.2 and 5.7 min was trapped at -70 °C in the cold trap and after 1.3 min reinjected onto the analytical column by flash-heating the cold trap to 180 °C. Detection was performed with a FID. Figure 2 shows the pre-column and analytical column chromatograms for these conditions. A considerable tailing of the solvent peak was observed, which is probably due to the 'dead' volume in the connecting materials and the dimensions of the injector, such as the internal diameter.

Optimization of the large volume injection

Large volume on-column injection onto a gas chromatographic column has been described extensively (Grob *et al.*, 1984, 1985; Munari *et al.*, 1985; Vreuls *et al.*, 1990). However, large volume injection onto a two-dimensional gas chromatographic system is not a common feature. Mol *et al.* (1993) showed the possibilities of the PTV injector as an interface. Chappel *et al.* (1993) described a system in which an on-column interface (OCI) was used for connecting a HPLC to a multidimensional gas chromatographic system. The columns were placed in series. The analytes were cryofocussed in a cold trap after monitoring the first column by using a split interface and a related detector.

We investigated the possibilities for large volume injection using the OCI in combination with the MUSIC system for the analysis of SM and D_8-SM.

SM and D_8-SM are medium boiling compounds in comparison with the solvent, therefore partially concurrent solvent evaporation (PCSE) conditions, as described by Munari *et al.* (1985), are most suitable for large volume injection. Furthermore, the interdependence of the column flows would not allow fully concurrent solvent evaporation. The large amount of evaporated solvent leads to 'backshooting' of the introduced compounds into the carrier tubing. Under PCSE conditions the large volume injection leads to a reconcentration of the analytes in the remaining solvent which forms a liquid layer in the retention gap. A number of parameters had to be optimized, such as injection pressure on the column, injection speed, evaporation speed, and injection temperature.

The injection pressure was set to 122 kPa and the oven temperature was kept constant at 70 °C during injection. Under these conditions the 'flooded' zone of the retention gap was estimated to be 10 - 11 cm/µl and the evaporation speed was calculated to be 27 µl/min when injecting with a speed of 40 µl/min during 10 min. After injection, the temperature of the oven was increased to 80 °C for 16 min in which time period the compounds elute from the retention gap and the pre-column. The fraction between 24.4 and 25.4 min was trapped at -70 °C in the cold trap and after heating the oven to 158 °C, the trap was subsequently flash heated to 180 °C upon which the analytes were reinjected (p_A = 78 kPa) onto the analytical column. Detection was performed with a FID, which resulted in a detection limit of 2 ng/ml of SM. Figure 3 shows the chromatograms obtained under these conditions.

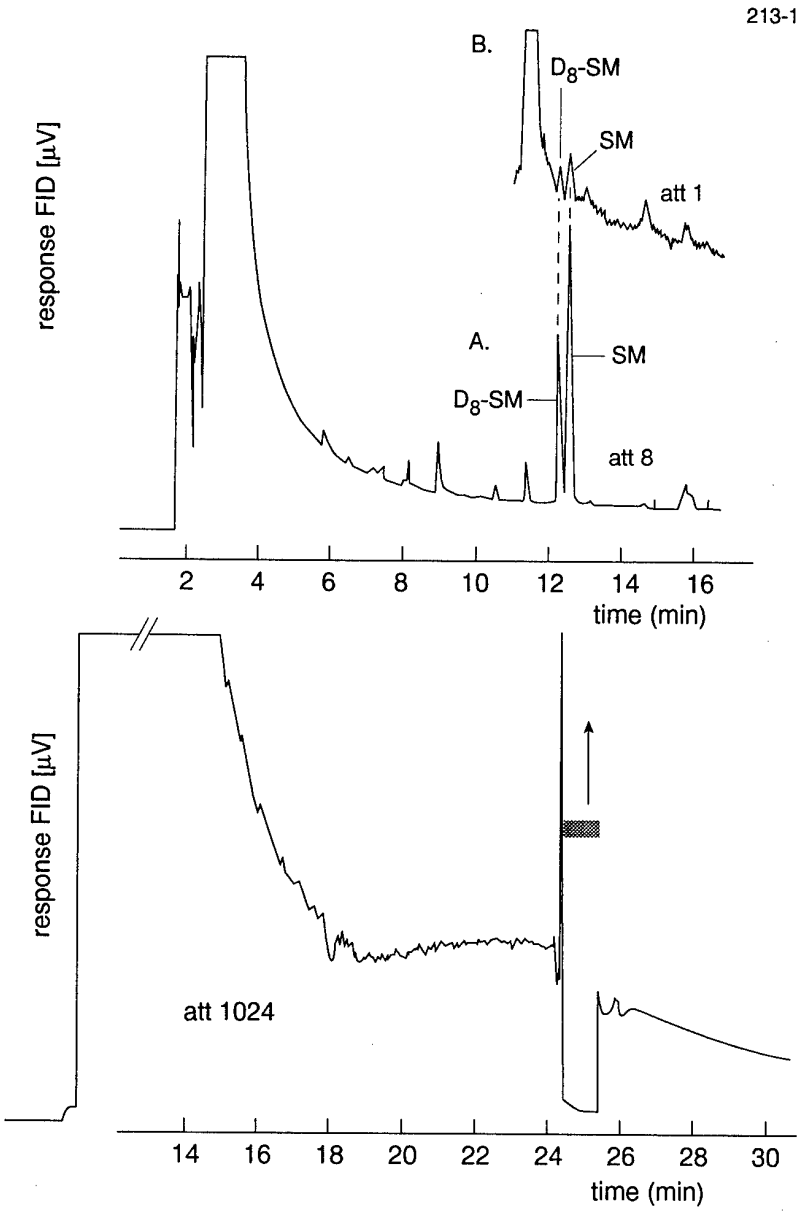

Figure 3. Chromatograms of a 400-µl on-column injection of a standard solution of SM and D_8-SM on GLC configuration 2. At the bottom the pre-column chromatogram is shown. The shaded area designates the fraction which was trapped and reinjected onto the analytical column. The top chromatograms show (A) a standard of SM and D_8-SM and insert (B), concentrations of SM and D_8-SM near the detection limit.

Optimization of the Early Solvent Vapor Exit (ESVE)
Due to tailing of the solvent peak on the pre-column, a large amount of solvent was trapped in the cold trap of the MUSIC system. This resulted in the introduction of a large amount of solvent on the analytical column, which had an adverse effect when using a sensitive detector such as an electron capture detector or a mass spectrometric detector. When a non-chemically bonded column would be used the result may even be disastrous, by stripping of the phase from the column. The ESVE (Grob *et al.*, 1989) in combination with a retaining pre-column is intended to remove most of the solvent before entering the analytical column, without loss of analyte. After installing the ESVE in our MUSIC sytem (see Figure 4) the optimum flow conditions changed dramatically in comparison with the conditions as described above.
Due to the pressure balance in the system a steady-state situation was established. However, by opening the ESVE valve this balance was severely distorted. The pressure drop between the top of the injector and the point to which the ESVE was connected, was increased from *ca.* 20 kPa to more than 100 kPa. Under these conditions, maintaining a constant evaporation speed and a liquid layer in the retention gap for the solvent effect is rather difficult. The conditions for injecting a sample of 400 µl were optimized. In order to maintain a flooded zone, which is necessary for partially concurrent solvent evaporation, the injection speed was increased from 40 to 185 µl/min. Under these conditions the evaporation speed was estimated to be 175 µl/min. The injection pressure for the pre-column was 182 kPa and the injection pressure for the analytical column 150 kPa; the oven temperature was set to 70 °C for 8 min, was subsequently increased to 140 °C at a rate of 10 °C/min, and kept constant for 12 min. The ESVE valve was opened during the 10 min of injection and subsequently closed after introduction of the last amount of sample. Obviously, the evaporation speed changes after closing this valve. Due to the back pressure of the MUSIC system (p_A = 150 kPa) the injection speed setting is critical. Next, the 22-m retention gap was reduced to 10 m. The fraction between 17.3 and 18.5 min was trapped in the MUSIC cold trap at -70 °C and after flow adjusting, flash-heated to 180 °C for reinjection onto the analytical column. Next, this configuration was combined with electron capture detection (ECD), in order to attain the required sensitivity. In this configuration we anticipated that the ECD could function routinely, since the sample reaching the detector is relatively clean due to ESVE and the two-dimensional chromatography. The detection limit for

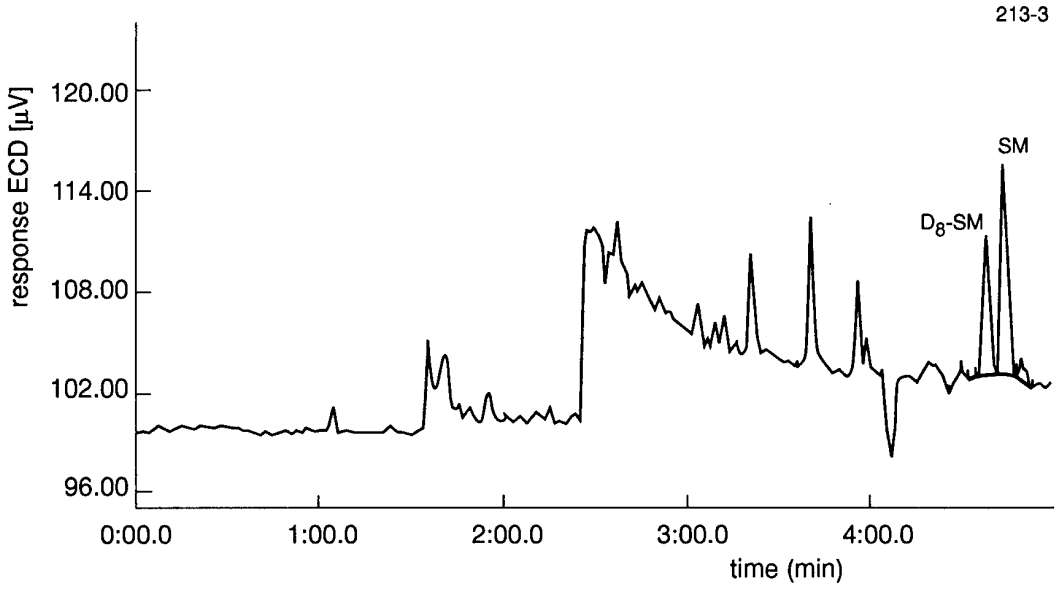

Figure 4. Detection of SM and D_8-SM in a 3-ml extracted blood sample, by on-column injection of 400 µl of ethyl acetate extract on GLC configuration 2. Please note the absence of a solvent peak.

SM was 10 pg for an injection of a 400-µl sample. Consequently, the limit of detection corresponds with 25 pg SM/ml blood without concentrating the ethyl acetate extract, which is 5-fold higher than the detection limit we aimed at. However, this might be sufficient for the purpose of our study. The within-day variability of the LV-GC-GC-ECD system appeared to be 2.8 % for SM (based on peak height), 6.5 % for D_8-SM, and 4 % for the SM/D_8-SM peak height ratio.

A cause for concern was the lack of reliability of the LV-GC-GC-ECD configuration in routine use. For no apparent reason no SM could be detected in some standard samples. Furthermore, the selectivity of the ECD in this configuration was not adequate: many small peaks were observed in the chromatograms, which made detection of SM at very low concentrations very difficult, in particular since the retention times of SM and D_8-SM were not very reproducible. A considerable effort was needed to keep the configuration going, which left hardly any time for analysis of samples. Therefore, it was decided not to continue along this line and to try another approach.

III.1.d. Gas chromatography with mass spectrometric detection

In view of the high sensitivity of mass spectrometric detection for SM, GC-MS seemed a promising approach for bioanalysis of SM. We chose not to make the configuration too complex in order to avoid technical problems as much as possible. By using an on-column autosampler the daily sample throughput was increased considerably. This autosampler has been used extensively in previous studies on the elimination pathways of C(±)P(±)-soman (Benschop and De Jong, 1990), and appears to be most reliable. Only 1-3 µl of sample can be injected in this way. This may be considered as a drawback of the method, since the extract is used in a very uneconomical way. However, the high sensitivity of the detection compensates for the small volume injection to such an extent that the required detection limit of *ca.* 5 pg SM/ml blood is within reach. Since we did not have experience with multidimensional chromatography in combination with MS detection, we decided not to spend time to develop such a combination. Due to the high selectivity of MS detection multidimensional chromatography is not deemed necessary. However, there is a considerable risk of contamination of the system without multidimensional chromatography.

Mass spectra of SM and D_8-SM are shown in Figures 5a and b. It is obvious that mass-fragment 109.1, which is $ClCH_2CH_2SCH_2^+$, is the most abundant fragment and therefore allows the most sensitive analysis.

An example of a GC-MS chromatogram of an extract of blood is shown in Figure 6. This figure shows that under these chromatographic conditions SM and D_8-SM are resolved, which is not necessary for MS-detection. Furthermore, the figure demonstrates the high selectivity of the MS-detection. The high selectivity in the TIC-chromatogram is the result of a rather narrow mass range of *m/z* 50-200. This range is scanned in 300 ms. Total analysis time is 4.5 min, which allows a high daily sample throughput. At 1.1 min after injection of the sample the filament is switched on. The bulk of the solvent has passed the detector by that time. In this way contamination of the detector is limited. Detection is started at 1.5 min after injection, which explains that there is no real injection peak in the chromatograms shown in Figure 6. When the concentrations of the analytes are too low for a full-scan of the aforementioned *m/z* range, detection of the major mass fragments 109 and 115 is chosen.

With some additional tuning of the mass spectrometric detection the absolute detection limit was improved further from 700 fg down to *ca.* 100 fg. For a 3-µl injection this corresponds with a detection limit of 33 pg SM/ml. The desired detection limit for SM in blood samples of 5 pg/ml can only be reached with this method, if the ethyl acetate extract is concentrated *ca.* sixfold under reduced pressure.

On the basis of the toxicokinetic pilot experiments it was anticipated that for most of the samples obtained in the toxicokinetic experiments, such a low detection limit would not be required since at 4 h after intravenous administration of a dose of SM corresponding with 1 LD50, the concentration of SM in blood was still around 1 ng/ml. However, volume reduction

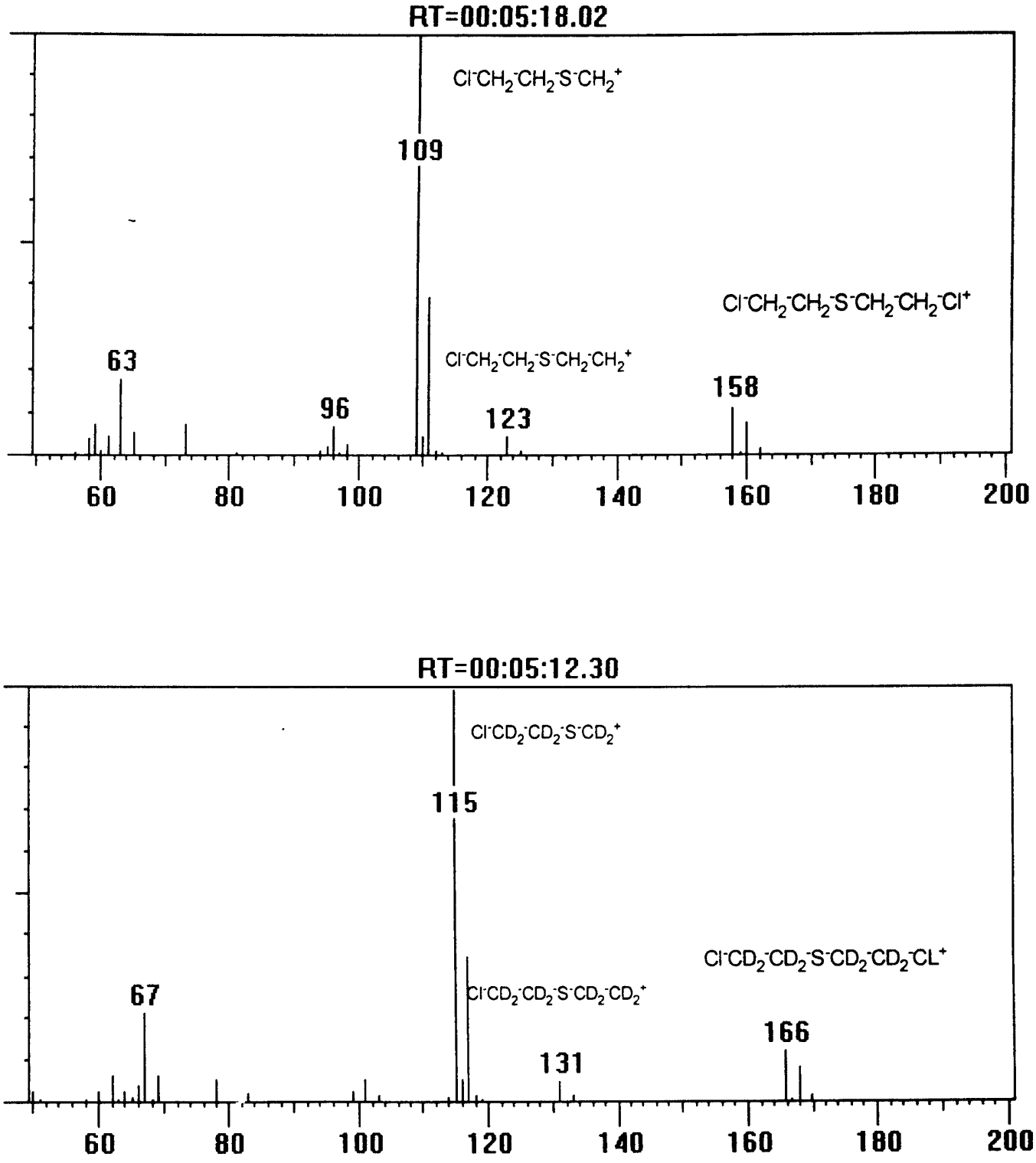

Figure 5. Mass spectra (ei) of (a) sulfur mustard, and (b) D_8-sulfur mustard.

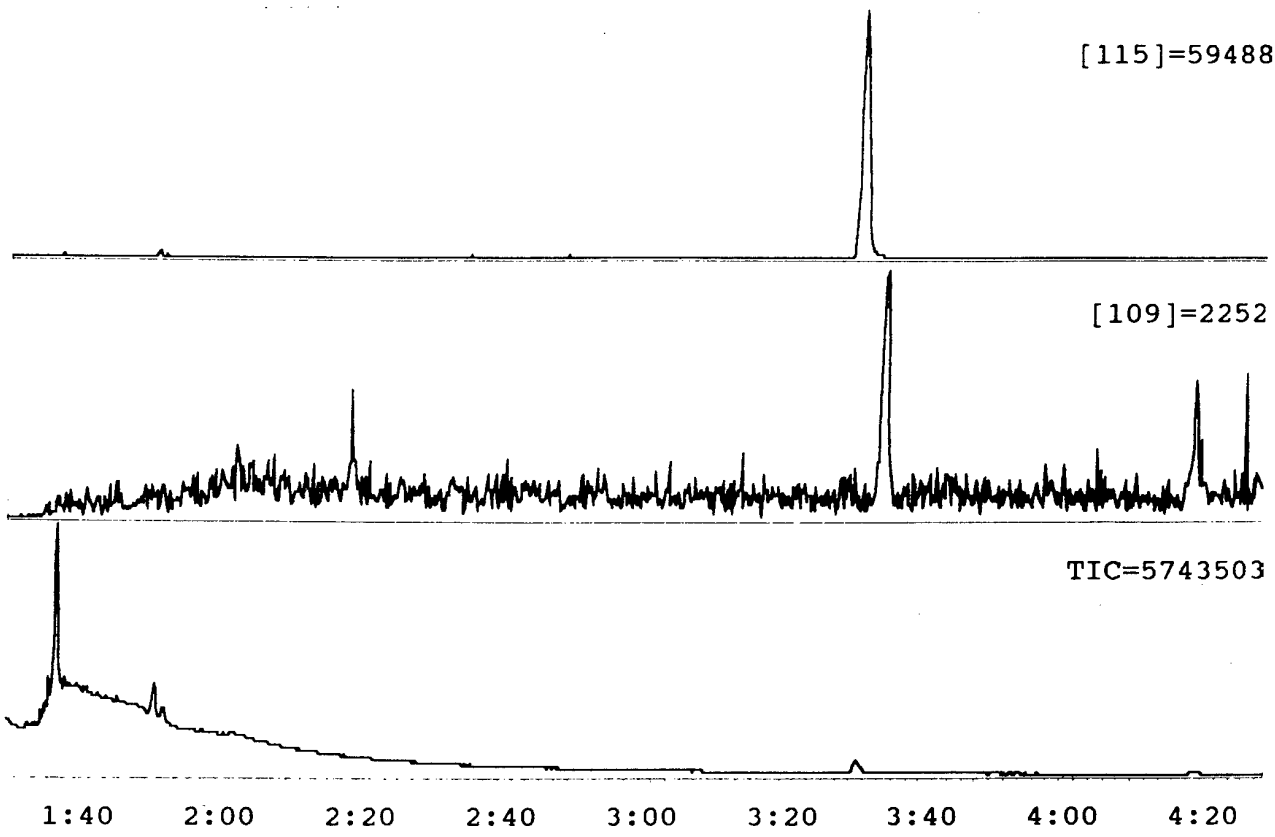

Figure 6. GC-MS chromatograms of an ethyl acetate extract of a blood sample drawn from a hairless guinea pig at 40 min after intravenous administration of 8.2 mg SM/kg. The peak detected at m/z 109 (middle chromatogram) is SM (55.7 ng/ml blood), the peak detected at *m/z* 115 (upper chromatogram) is D_8-SM (525 ng/ml blood). The lower chromatogram shows the total ion current (TIC).

of the ethyl acetate extract will be necessary for samples taken at time points later than 4 h, or if lower doses of SM are administered.

Unfortunately, problems arose during the application of this configuration in the toxicokinetic studies. The linearity between the injected amount and peak height was lost in the range of 1-100 pg, resulting in a detection limit of *ca.* 10 pg and irreproducible results. These problems appeared to be mainly of a chromatographic nature. The retention gap was heavily contaminated, with droplets visible in the capillary. Furthermore, the performance of the analytical column had deteriorated. After renewal of the retention gap and analytical column, and some fine-tuning of the MS, linearity and sensitivity were restored. Due to the high selectivity one tends to forget that the samples are not as clean as they seem to be by the appearence of the chromatograms. Lipids will be extracted from the sample with ethyl acetate, which amounts to about 5 mg/ml blood. As a consequence, *ca.* 5 µg of lipids are injected upon each 1-µl injection into the GC-system, which form a "stationary phase' in the retention gap and subsequently also in the analytical column. This leads to deterioration of chromatographic

performance. A procedure for preventive maintainance was established, based on replacement of the retention gap after injection of 40 biological samples. Unfortunately, this procedure appeared to be insufficient.
In order to reduce the contamination of the chromatographic system two approaches were chosen. Firstly, additional clean-up of the extract using solid phase extraction cartridges was studied. For a high recovery this additional step should be as simple as possible. It was attempted to get rid of the lipids by leading the ethyl acetate extract over a Florisil® cartridge, anticipating that SM and D_8-SM would not be retained on the cartridge.
Ethyl acetate extracts of guinea pig blood were led over a SepPak Florisil® cartridge. The lightly yellow extract was decolorized by this action. Furthermore, upon evaporation of the ethyl acetate eluate from the cartridge, the mass of the residue was reduced by about 50 % in comparison with the ethyl acetate extract not led over Florisil®. By spiking SM and D_8-SM into ethyl acetate extracts from blood it was shown that they were not retained on the Florisil® cartridge. Since this additional clean-up step of the ethyl acetate extract from blood seemed to be a promising approach, it was temporarely incorporated into our analytical procedure. However, as was anticipated, this additional clean-up was not sufficient to prevent serious contamination of the chromatographic system when analyzing large numbers of samples. As a consequence, the Florisil clean-up step was omitted from the analytical procedure shortly after it was introduced.
As a second approach it was attempted to reduce the contamination of the chromatographic system by using thermal cold trap (TCT) injection. This injection technique was successfully applied in previous toxicokinetic studies of the nerve agents soman and sarin (Benschop and Van Helden, 1993). TCT-injection has two important advantages: it allows large volume injection and on-line sample clean-up by choosing appropriate desorption conditions. Furthermore, TCT-injection can be automated by using a thermodesorption autosampler (TDAS) which we have at our disposal. Conditions for TDAS-GC analysis of SM were already known in our laboratory from other applications and were adapted for our specific situation. In view of the low absolute detection limit of the GC-MS configuration for SM (*ca.* 100 fg) and since a volume of ethyl acetate extract equivalent with 0.5 ml blood could be brought onto the Tenax material, a detection limit for SM in blood below 5 pg/ml could be reached on a routine basis with this configuration.
The TDAS-GC-MS configuration appeared to be fairly reliable in routine bioanalysis.

III.1.e. Gas chromatography with (pulsed-)flame photometric detection

Although the TDAS-GC-MS configuration had proven to be reasonably reliable in general, the analysis remained an important bottleneck in the project. This was due to the large numbers of samples that were generated in each animal experiment, which tended to pile up as analytical problems occurred. Therefore, we intended to reduce the workload of the TDAS-GC-MS configuration by setting up an additional GC-configuration for measurement of samples which contained relatively high concentrations of SM, i.e., those taken in the first few minutes after i.v. administration. For this purpose we first set up a TCT-GC-FPD configuration. The absolute detection limit (S/N=3) for SM with the FPD was *ca.* 200 pg, which was not spectacular. However, the TCT injector allowed injection of the equivalent of several hundreds of microliters of sample, which compensated for the lower sensitivity of the FPD to some extent. Furthermore, the FPD is extremely selective, which is an important advantage of this detector. After optimization of the TCT-GC-FPD configuration we reached a limit of determination (S/N=10) of 1 ng SM/ml blood, when loading 400 µl of ethyl acetate extract onto Tenax, which would allow us to measure the samples taken at approximately $\leq$ 1 h after i.v. administration of SM at a dose corresponding with 1 LD50.
As mentioned in paragraph III.1.b, the pulsed flame photometric detector (PFPD) has been recently introduced (Amirav and Jing, 1995). Whereas the operation of a standard flame photometric detector is based on a continuous flame for the generation of flame

chemiluminescence, the PFPD uses a pulsed flame for these purposes. The detection selectivity of the PFPD is enhanced by the additional dimension, i.e., the light emission time, and the ability to separate in time the emissions of carbon species from that of sulfur or various other atoms. Furthermore, the detection sensitivity is higher than that of the FPD, due to a reduced flame background noise, an increased signal due to the higher brightness of the pulsed flame, and the use of broad-band filters instead of interference filters.
Upon arrival, our Varian Star 3400 CX gas chromatograph with Septum Programmable Injector (SPI) and PFPD, was brought up to specifications. An example of a chromatogram obtained after injecting a standard of SM and D_8-SM in ethyl acetate is shown in Figure 7. The absolute detection limit for SM was determined to be *ca.* 10 pg. For a 1-µl injection this corresponds with an SM concentration 10 ng/ml blood. With the SPI, injection volumes of 3 µl appeared to be feasible without significant peak broadening. Injection of 3 µl of sample reduces the detection limit further, to *ca.* 3.3 ng/ml blood. Obviously, a larger injection volume will be accompanied by a more rapid contamination of the system. This chromatographic configuration was applied

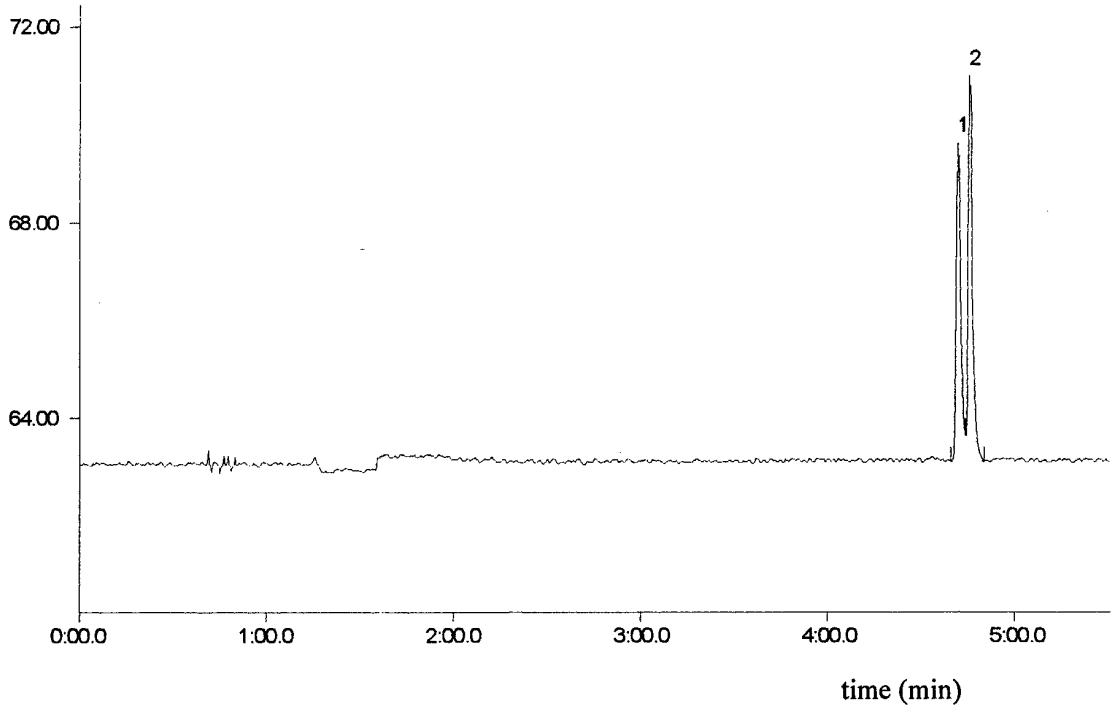

Figure 7. Chromatogram obtained after injection of 1 µl of an ethyl actete solution containing D_8-SM (peak 1, 210 ng/ml) and SM (peak 2, 200 ng/ml) into the SPI-GC-PFPD configuration under the conditions described in paragraph II.2.

successfully to the analysis of SM in tissue samples, taken shortly after i.v. administration of a dose corresponding with 1 LD50.
In order to be able to inject larger sample volumes while reducing contamination of the system, a TCT-injector was installed on the Varian Star 3400 CX gas chromatograph with PFPD. Up to 400 µl of sample in ethyl acetate were brought onto Tenax. An absolute detection limit for SM of *ca.* 50 pg (S/N=3) was accomplished routinely with this configuration, which corresponds with 125 pg/ml. This is a marked improvement in comparison with on-column (SPI) injection (10 or 3.3 ng/ml for 1- or 3-µl injection, respectively). This chromatographic configuration was succesfully applied to the analysis of SM in tissue samples.

III.2 ANALYSIS OF THE MAJOR ADDUCT OF SULFUR MUSTARD TO DNA

III.2.a. Optimization of the immunoslotblot assay

Several attempts have been made to increase the sensitivity and reproducibility of the immunoslotblot assay, and to simplify the procedure. These attempts have resulted in some significant improvements with respect to the DNA isolation procedure, the binding of DNA to the nitrocellulose filter, as well as the measurement of chemiluminescence. The results are described below.

DNA isolation

Originally, DNA was isolated according to the laborious phenol extraction method. A commercial preparation (Pure-gene DNA isolation kit , Biozym) became available which enabled a much faster DNA-isolation procedure and which could also be applied to our tissue samples without any loss or destruction of adducts from the DNA. In this procedure, described in section II.7, DNA was suspended in a tenfold diluted TE buffer in the final step, instead of the TE buffer. The resulting decrease in ionic strength facilitated the unwinding of the DNA by the subsequent thermal denaturation which is required for application of the immunoslotblot assay.

DNA binding to nitrocellulose filter in the immunoslotblot assay

After denaturation of the DNA, the DNA solution was diluted in PBS to a final concentration of 5 µg/ml. Next, 200 µl of the DNA solution was blotted onto the nitrocellulose filter, washed with PBS, and dried on air. Next, the DNA was crosslinked to the filter with by UV irradiation (50 mJ/cm^2) with the GS Gene Linker UV Chamber (Bio-Rad Laboratories, Netherlands). The rest of the procedure was as described previously.

Pre-flashing of photographic films

In order to improve the blackening characteristics of the photographic films these were pre-illuminated with a Sensitize Pre-Flash Unit (Amersham Netherlands BV) at a distance of 75 cm from the light source, just prior to exposure to the chemiluminescent light from the nitrocellulose filters. In later experiments, using films of Kodak (X-Omatic) instead of those of Amersham, it appeared that pre-illumination was not necessary.

Direct measurement of chemiluminescence with a luminometer

In the course of this study three luminometers (Microlumat 2B96B, EG & G Benelux BV, Nieuwegein, The Netherlands; TopCount, Packard Instrument Company, USA; and 1450 MicroBeta Trilux Luminescence Counter, EG & G Wallac, Breda, The Netherlands) were tested with respect to their suitability to measure directly the chemiluminescence in the immunoslotblot assay. It appeared that chemiluminescence can be measured with all three luminometers with at least the same sensitivity and reproducibility as with photographic film in combination with the densitometer. The MicroBeta luminometer was procured since this apparatus was judged to be the most practical for use with immunoslotblots. In daily use the luminometer appears to be more convenient and less time-consuming than photographic films. It can be applied over a wide range of chemiluminescence intensity.

In an experiment in which we repeated the immunoslotblot assay it appeared that differences in adduct levels in the same DNA samples were measured from day-to-day relative to the adduct levels in the control DNA samples. It appeared that at least one freezing/thawing cycle of the samples after thermal denaturation made the measurements much more reproducible.
It also appeared that the chemiluminescence signal strongly depends on the amount of DNA spotted on the filter, as is shown in Figure 8. Consequently, it was decided to blot 1 µg of DNA for each sample from that moment on.

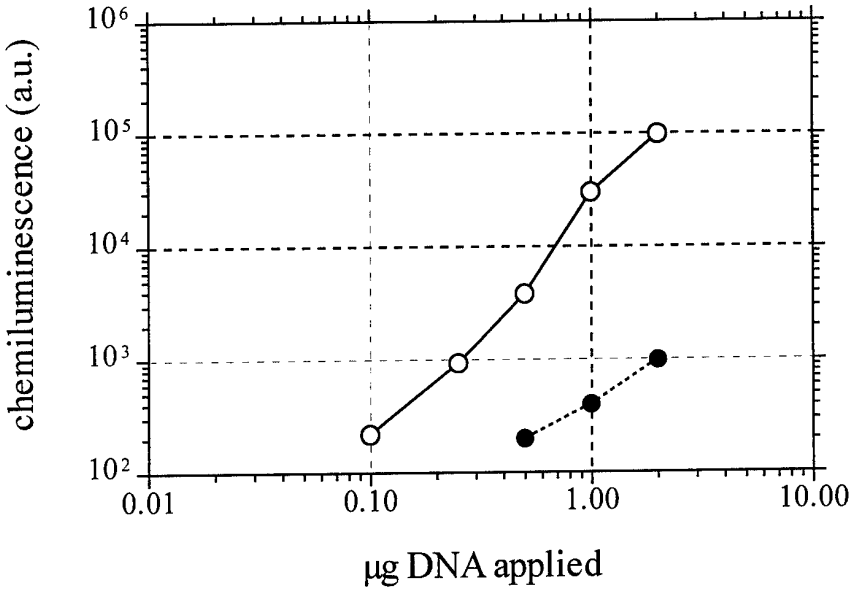

Figure 8. Immunoslotblot assay of 7-SM-gua in ds-ct-DNA exposed to 0 (●) or 2.5 nM SM (**O**, 30 min 37 °C), using the Wallac MicroBeta luminometer.

Preparation of representative tissue samples

In the course of the study we observed a large variation in the tissue adduct levels between animals. This could, of course, be due to biological variation within this outbred hairless guinea pig strain. However, another explanation for this phenomenon could be that the DNA adducts are not homogeneously distributed within the tissues studied. In that case, the tissue as a whole would have to be homogenised before taking a sample for analysis, preferably in such a way that both intact SM as well as SM-adducts could be detected. For relatively large tissues such as liver and lung it appeared to be difficult to find a procedure to homogenize the tissue as a whole in a short time period, thus avoiding or at least limiting degradation of SM in the sample. Attempts to homogenize frozen material with a micro-dismembrator failed, because only small amounts of frozen material could be pulverized in one run, whereas the temperature increased considerably during the process. The best approach appeared to be homogenization of the tissue with an ultrathurrax mixer immediately after dissection of the animal. Subsequently, the homogenate was divided into two portions, i.e., one for analysis of intact SM and one for quantification of the adduct to guanine. These samples were kept at –70 °C until analysis. Interestingly, large variations in adduct concentrations between animals were still observed in the homogenates from the tissues as a whole. Consequently, biological variation seems to be a more likely explanation for the difference in tissue adduct concentration than inadequate sampling procedures.

7-SM-gua in blood samples of hairless guinea pigs

Considerable difficulties were encountered in our attempts to isolate DNA from blood samples of the hairless guinea pig. This was presumably due to an inproper ratio of the amount of anticoagulant (heparin and/or EDTA) as suggested by the presence of a precipitate after thawing of the frozen blood samples. Some modifications were applied to the isolation method in order to rescue the samples that were already collected. In some cases we used the Boehringer DNA isolation kit to isolate DNA from blood. When applied on human blood, the DNA isolation kits of both Biozym and Boehringer yielded reasonable amounts of DNA at an acceptable purity. Application of the Boehringer DNA isolation kit to blood samples of the hairless guinea pig instead of that of Biozym resulted in some improvement, although insufficient amounts of DNA were isolated from all several samples. However, DNA could be obtained from all blood samples when lysis of the erythrocytes was performed with 0.1 M acetic acid, albeit with a low

purity. Nevertheless, these samples were assayed in the immunoslotblot assay. Results are presented in Figure 9.

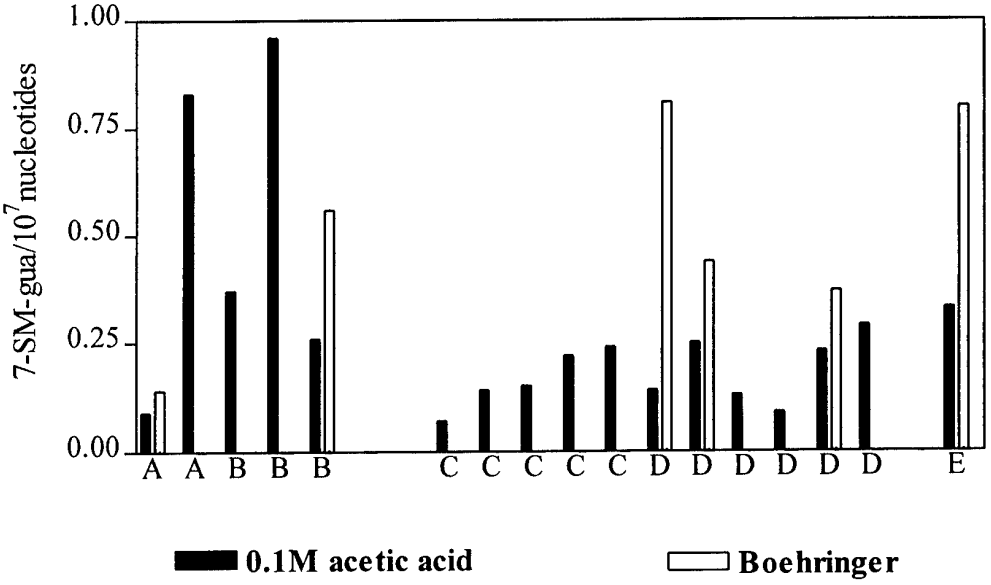

Figure 9. 7-SM-gua in blood of hairless guinea pigs after nose-only exposure to 1 (5 animals)or 3 LCt50 (11 animals) SM vapor in air or after 1 LD50 of SM i.v. (1 animal). Blood was collected at 2 and 4 h after nose-only exposure and at 3 h after i.v. administration of SM, and subsequently frozen. A: 2 h after 1 LCt50, B: 4 h after 1 LCt50, C: 2 h after 3 LCt50, D: 4 h after 3 LCt50, E: 3 h after 1 LD50. After thawing, the precipitate present in the blood samples, was dissolved in either the Boehringer RBC lysis solution or 0.1 M acetic acid. In both cases, the pelleted white blood cells were further processed with the Boehringer DNA isolation kit and the DNA was assayed in the immunoslotblot assay.

The results indicate that in all cases significant but only low levels of 7-SM-gua are obtained. The data further suggest that treatment with 0.1 M acetic acid induces some loss of 7-SM-gua. In addition, we attempted to prevent coagulation by varying the amount of EDTA in the collected blood, but sofar without success. The least unsatisfactory results were obtained when lysing the red blood cells immediately after collection of the blood.

III.2.b. Immunofluorescence microscopy

Immunofluorescence microscopy was successfully applied for the qualitative and quantitative analysis of 7-SM-gua within the context of Grant No. DAMD17-88-Z-8022 (Benschop, 1991) and Cooperative Agreement DAMD17-92-V-2005 (Benschop and Van der Schans, 1995). The method appeared to be of particular value for samples with a very large concentration of DNA adducts. Frequently, DNA cannot be isolated from such samples, thus hampering the immunoslotblot assay. Therefore, we applied immunofluorescence microscopy to samples in which we anticipated high adduct levels, such as skin samples taken from the percutaneous toxicokinetic experiments and the efficacy tests of topical skin protectants. The method as developed previously (Benschop, 1991; Benschop and Van der Schans, 1995) was applied as such in the current study.

III.3 THE 96-h LD50 (I.V. BOLUS) OF SULFUR MUSTARD IN THE HAIRLESS GUINEA PIG

III.3.a. Pilot experiments

Hardly any LD50 values for SM are documented. In an early article by Anslow *et al.* (1948), values for i.v., s.c., and percutaneous 15-day LD50s are mentioned for mice, rats and rabbits. As far as the i.v. LD50 was concerned, the indicated values for mice, rats and rabbits were 8.6, 0.7 and 2.7 mg/kg, respectively. Maisonneuve *et al.* (1993) reported a 14-day LD50 for SM in rats of 3.8 mg/kg.

Usually, LD50 values are based on 24-h lethality scores. However, in view of the quiescent period of 3-12 h after SM intoxication, we anticipated that a 24-h period will be too short to obtain a realistic value for the LD50. Therefore, we proposed to use 96-h lethality scores to determine the LD50 value. During this period the animals had access to water and food *ad libitum*. To reduce the suffering of the animals during this long time period an analgesic was used. Approximate times of death of the animals were registered, which would allow calculation of LD50 values for other periods than 96 h, in case the latter time period would prove to be too long.

Because of the divergent values reported in literature for the various species and complete lack of toxicity data on the hairless guinea pig, some pilot-experiments for dose-range finding purposes were necessary. In a first pilot-experiment doses of 1, 2, 3, 4, or 5 mg/kg were i.v. injected in anesthetized animals (2 animals per dose) using a stock solution of 50 mg/ml of SM in isopropyl alcohol. Just before administration, an adequate portion of this solution was diluted with saline, in such a way that administration of 1 ml/kg body weight of this solution resulted in the doses mentioned above. After administration of SM, survival times were assessed by keeping them one to a cage and recording (breathing) movements using an ultrasonic detection device. Twice a day during the next 5 days the animals were treated by the long-lasting analgesic Temgesic®. During this 5-day observation period the animals had access to food and water *ad libitum*. Within the 96-h time period there was no mortality except for one animal in the 3 mg/kg group which died shortly after the administration of SM. After 96 h one animal in the 4 mg/kg group died. The data of this first pilot experiment are gathered in Table 2.

Table 2. Weight changes and mortality in the first pilot-experiment for the determination of the 96-h i.v. LD50 of SM in the male hairless guinea pig.

Guinea pig #	SM dose (mg/kg, i.v.)	Day 0 Weight		Day 2 Weight		Day 5 Weight	
		(g)	%	(g)	%	(g)	%
1	1	447	100	433	96.9	459	102.7
2	1	491	100	470	95.7	476	96.9
3	2	458	100	451	98.5	442	96.5
4	2	543	100	532	98.0	537	98.9
5	3	507	100	†			
6	3	534	100	498	93.3	490	91.8
7	4	497	100	472	95.0	†	
8	4	513	100	474	92.4	430	83.8
9	5	528	100	500	94.7	376	71.2
10	5	487	100	456	93.6	433	88.9

† = deceased

In the second pilot-experiment doses in the range of 7-15 mg/kg were administered to the animals. The results are shown in Table 3.

Table 3. Weight changes and mortality in the second pilot-experiment for the determination of the 96-h i.v. LD50 of SM in the male hairless guinea pig.

Guinea Pig #	Dose SM (mg/kg)	Time 0 h Weight (g)	%	Time 24 h Weight (g)	%	Time 48 h Weight (g)	%	Time 72 h Weight (g)	%	Time 96 h Weight (g)	%	Survival time (h)
11	7	398	100	346	86.9	342	85.9	324	81.4	294	73.9	> 96
12	7	424	100	382	90.1	369	87.0	355	83.7	320	75.5	> 96
13	9	382	100	332	86.9	†						36.8
14	9	348	100	301	86.5	277	79.6	283	81.3	252	72.4	> 96
15	11	374	100	†								5.7
16	11	411	100	†								0.75
17	13	420	100	†								20.2
18	13	368	100	†								6.9
19	15	398	100	†								6.9
20	15	426	100	†								3.3

† = deceased

b. The 96-h LD50 of sulfur mustard

In view of the results of the pilot-experiments, the doses chosen for the actual LD50 experiment were 7.5, 8, 8.5, 9, and 9.5 mg/kg. Each dose was administered to 6 hairless guinea pigs. The results are presented in Table 4.

Table 4. Number and percentage of dead animals per dosing group, 96 h after i.v. bolus administration of SM to anesthetized hairless guinea pigs.

Dose of SM (mg/kg, i.v.)	Number of dead animals at 96 h/total number	Percentage deaths
7.5	1/6	16.6
8.0	4/6	66.6
8.5	3/6	50
9.0	4/6	66.6
9.5	6/6	100

The 96-h LD50 was calculated from the mortality data by probit analysis (Litchfield and Wilcoxon, 1949). The results of the probit analysis are presented in Table 5 and Figure 7. From these data the intravenous bolus 96-h LD50 of SM in the hairless guinea pig was calculated to be 8.2 mg/kg (95 % confidence limits 7.1-8.8 mg/kg).

Table 5. LD10, LD30, LD50, LD70 and LD90 (96-h) values with 95 % confidence limits, for i.v. bolus administration of SM to anesthetized hairless guinea pigs, calculated via probit analysis[a].

LD	mg/kg	95 % confidence limits (mg/kg)
10	7.0	3.6 - 7.7
30	7.7	5.4 - 8.2
50	8.2	7.1 - 8.8
70	8.7	8.2 - 10.6
90	9.5	8.8 - 15.8

[a] Probit equation: probit = 19.6*log (dose sulfur mustard) - 12.9

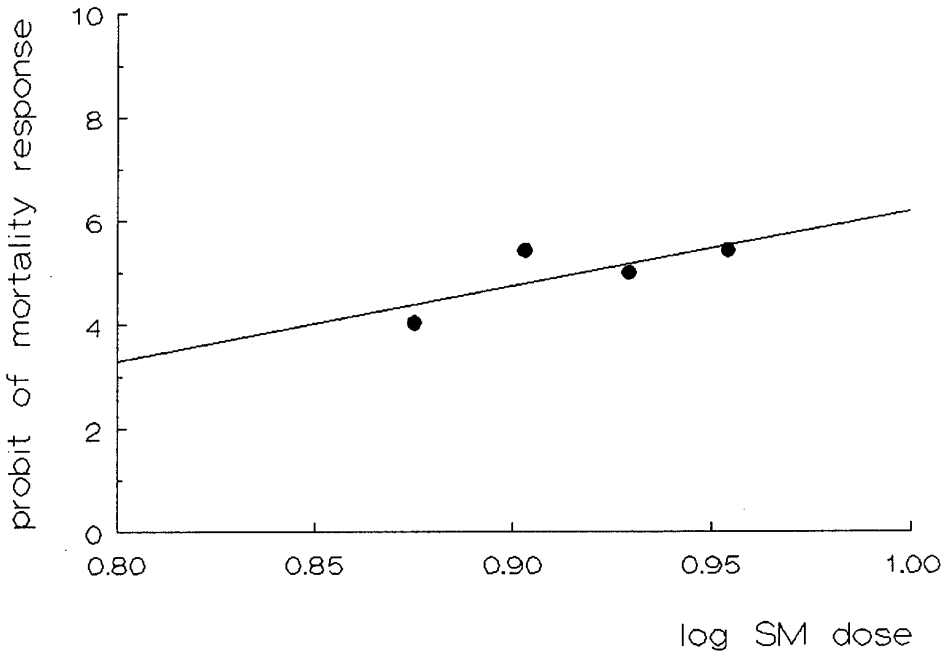

Figure 10. Probit of mortality of hairless guinea pigs, 96 h after intravenous bolus administration of SM to the anesthetized animals, versus the administered dose of SM.

III.4 TOXICOKINETICS OF SULFUR MUSTARD IN ANESTHETIZED HAIRLESS GUINEA PIGS AFTER INTRAVENOUS BOLUS ADMINISTRATION OF A DOSE CORRESPONDING WITH 1 LD50

III.4.a. Toxicokinetics of sulfur mustard in blood

Male hairless guinea pigs were anesthetized, the jugular vein was made accessible, and a carotid canulla was inserted. SM was injected into the jugular vein in a dose of 8.2 mg/kg, which corresponds with 1 LD50 (96-h). Blood samples were drawn via the carotid canulla just before administration of the toxicant (time 0) and at 0.5, 1, 2, 4, 10, 15, 20, 30, 40, 60, 90, 120, 180, 240, 300 and 360 min after administration. The size of the samples ranged from 0.1 ml in the first samples up to 3 ml for the final two sampling times. In order to avoid too much strain on the individual animals, the sampling times were divided between two animals. Theoretically, 12 animals would be needed to obtain 6 values for each time point. However, since for some of the samples the analysis failed, additional experiments had to be performed. A total number of 16 hairless guinea pigs was used to complete this task.

The blood samples were extracted with ethyl acetate to which the internal standard was added. The extracts were analyzed with GC configurations 3 and 4.

The mean concentrations with standard error of the mean are presented in Table 6, whereas the concentrations of SM in blood measured in the individual animals are presented in Table 7. The mean concentration-time course is shown in Figure 11. Toxicokinetic parameters derived from the concentration-time course are presented in Table 8.

Table 6. Mean concentration in blood (ng/ml ± s.e.m.) of SM in anesthetized hairless guinea pigs at various time points after i.v. administration of 1 LD50 (8.2 mg/kg) of SM.

Time (min)	Concentration of SM (ng/ml blood ± s.e.m.)	
0	n.d.	(n=12)
0.5	3600 ± 900	(n=6)
1	2200 ± 700	(n=6)
2	850 ± 250	(n=6)
4	160 ± 80	(n=6)
10	4.6 ± 2.0	(n=7)
15	2.5 ± 1.7	(n=6)
20	4.2 ± 2.0	(n=6)
30	1.6 ± 0.5	(n=6)
40	1.1 ± 0.6	(n=6)
60	0.8 ± 0.2	(n=7)
90	1.3 ± 0.6	(n=6)
120	1.4 ± 0.2	(n=6)
180	1.1 ± 0.4	(n=6)
240	0.7 ± 0.6	(n=5)
300	0.5 ± 0.3	(n=6)
360	0.6 ± 0.1	(n=5)

n.d. = not detectable (< 5 pg/ml)

Table 7. Concentrations in blood of SM (ng/ml) in individual anesthetized male hairless guinea pigs at various time points and after intravenous administration of a dose of 8.2 mg/kg, which corresponds with 1 LD50 (96-h).

	Concentration of SM in blood (ng/ml) Hairless guinea pig #															
Time (min)	A1 (590)[a]	B1 (590)[a]	A2 (739)[a]	B2 (874)[a]	C2 (863)[a]	D2 (866)[a]	A3 (618)[a]	B3 (642)[a]	C3 (671)[a]	D3 (677)[a]	A4 (682)[a]	B4 (720)[a]	C4 (670)[a]	D4 (706)[a]	A5 (660)[a]	B5 (710)[a]
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.
0.5	7169	*	2459	*	2522	*	4472	*	4346	*	615	*	*	*	*	*
1	*	2421	*	1205	*	1592	*	5169	*	447	*	2527	*	*	*	*
2	2044	*	196	*	650	*	489	*	1247	*	478	*	*	*	*	*
4	*	12.5	*	16.0	539	*	*	216	*	142	*	39.1	*	*	*	*
10	12.5	*	f.a.	*	*	f.a.	1.4	*	*	f.a.	2.5	1.3	11.7	1.0	*	1.9
15	*	f.a.	*	f.a.	*	f.a.	*	0.6	*	f.a.	f.a.	10.8	1.2	n.d.	1.1	1.4
20	5.7	*	2.2	*	3.0	*	f.a.	*	0.3	*	f.a.	*	0.9	13.2	*	*
30	*	0.9	*	0.7	3.1	*	*	3.3	0.9	*	*	0.5	*	*	*	*
40	4.1	*	0.5	*	*	0.4	1.0	*	*	0.4	0.1	*	*	*	*	*
60	*	1.7	*	0.2	*	0.7	*	1.4	0.9	0.7	*	0.2	*	*	*	*
90	4.2	*	0.4	*	0.9	*	0.7	*	1.0	*	0.7	*	*	*	*	*
120	*	1.1	*	1.7	1.9	*	*	1.1	1.4	*	*	0.9	*	*	*	*
180	0.8	*	2.2	*	1.3	*	*	1.9	0.3	*	*	0.1	*	*	*	*
240	*	2.8	*	f.a.	*	f.a.	0.3	*	*	f.a.	f.a.	*	f.a.	n.d	n.d.	0.2
300	*	f.a.	*	2.0	*	0.1	f.a.	*	*	0.3	f.a.	*	n.d.	f.a.	0.4	0.4
360	f.a.	*	0.5	*	f.a.	*	0.3	*	*	f.a.	0.1	*	*	*	2.2	n.d.

[a] Weight in grams

n.d. = not detectable (< 5 pg/ml)

f.a. = failed analysis

* = not determined

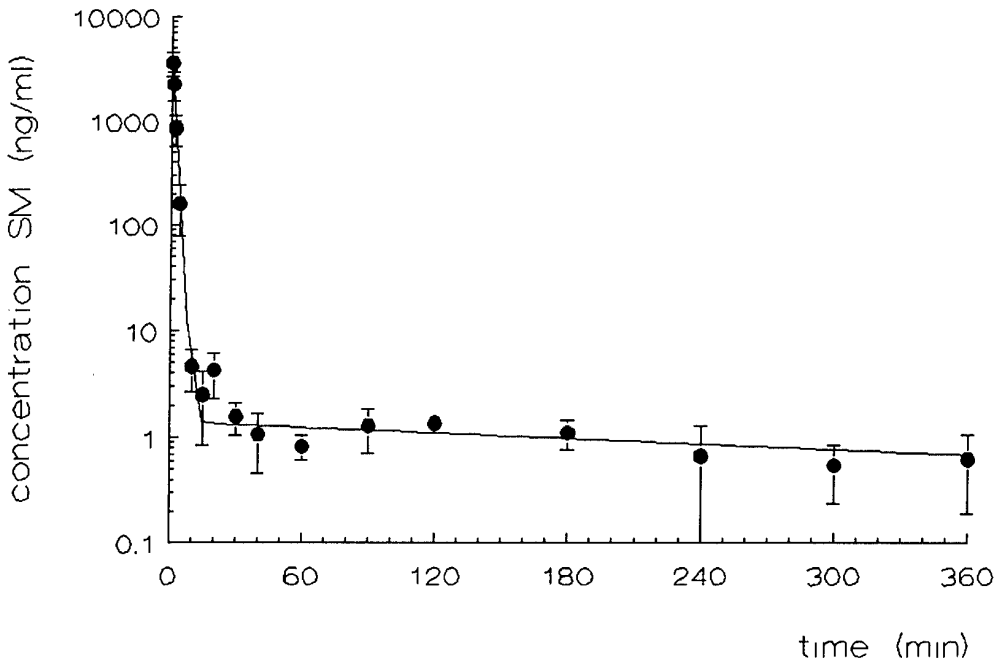

Figure 11.Mean concentration-time course of SM in the blood of anesthetized hairless guinea pigs after i.v. administration of 8.2 mg SM/kg, which corresponds with 1 LD50 (96-h).

It is clear from Figure 11 and the parameters in Table 8 that the toxicokinetics of SM in the hairless guinea pig are characterized by a very rapid initial phase, which can be designated as the distribution phase, and a very slow terminal phase, which can be designated as the elimination phase.

Table 8. Toxicokinetic parameters[a,b] for SM in anesthetized hairless guinea pigs after intravenous bolus administration of 8.2 mg/kg, which corresponds with 1 LD50 (96-h).

Number of exponents		2
F-ratio		0.41[c]
A	($ng.ml^{-1}$)	4665
B	($ng.ml^{-1}$)	1.41
α	(min^{-1})	0.78
β	(min^{-1})	0.0020
AUC	($ng.min.ml^{-1}$)	6639
C_0	($ng.ml^{-1}$)	4666
$k_{1,2}$	(min^{-1})	0.082
k_{el}	(min^{-1})	0.70
$k_{2,1}$	(min^{-1})	0.0023
$t_{½dis}$	(min)	0.88
$t_{½el}$	(min)	342
V_1	($l.kg^{-1}$)	1.76
V_{dss}	($l.kg^{-1}$)	65.2
Cl	($l.min^{-1}.kg^{-1}$)	1.24
MRT	(min)	52.7

[a] The concentration of sulfur mustard at time t is described by: $[SM] = A*e^{-\alpha t} + B*e^{-\beta t}$

[b] Abbreviations used: AUC, area under the curve; C_0, retrapolated concentration in the central compartment at time 0; $k_{1,2}$, rate constant of transfer from compartment 1 to compartment 2; k_{el}, rate constant of elimination; $k_{2,1}$, rate constant of transfer from compartment 2 to compartment 1; $t_{½disi}$, distribution half-life; $t_{½el}$, terminal half-life; V_1, volume of the central compartment; V_{dss}, volume of distribution under steady-state; Cl, total body clearance; MRT, mean residence time.

[c] Critical value $F_{2,10} = 4.10$ (p=0.05)

III.4.b. Distribution of intact sulfur mustard to tissues

Concentrations of intact SM in various tissues were measured at four time-points after intravenous administration of a dose of SM corresponding with 1 LD50. The tissues were liver, lung, spleen, bone marrow and abdominal fat. The time-points were 3 and 10 min after intravenous administration of SM, as well as halfway and at the end of the toxicokinetic experiment, i.e., 180 and 360 min after administration, respectively. Extracts from homogenzied tissues in ethyl acetate were analyzed with GLC configuration 5 or 6 (*cf.* Section II. 2).The results are presented in Table 9, and in Figures 12 and 13.

SM was present in detectable concentrations in all of the samples. At 3 and 10 min after intravenous administration the concentrations of intact SM in the lung, spleen and bone marrow are higher than in blood. In the liver the concentration SM is about equal to that in blood at 3 min, but has increased about 3-fold at 10 min after administration.

At 180 and 360 min the concentration of SM in lung is approximately equal to that in blood, whereas the concentrations in spleen, bone marrow and liver are one to two orders of magnitude higher then that in blood. These results indicate a substantial partitioning of SM from the blood to the tissues.

Table 9 Concentration (ng/g) of intact SM in various tissues sampled at 3, 10, 180 or 360 min after intravenous administration of 8.2 mg/kg SM to anesthetized hairless guinea pigs, which corresponds with 1 LD50 (96-h). Mean values with s.e.m. (n=4, unless stated otherwise) are also presented.

Animal #	Time (min)	Concentration SM in tissue (ng/g)				
		Liver	Lung	Spleen	Marrow	Fat
SM1	3	765	17100	2120	2040	f.a.
SM2	3	240	4240	1600	114	f.a.
SM5	3	165	430	170	450	f.a.
SM13	3	235	47	235	55	f.a.
Mean ±	s.e.m.	350 ± 140	5400 ±4000	1030 ± 490	660 ± 470	
SM3	10	905	840	280	45	f.a.
SM4	10	1100	2530	125	26	f.a.
SM6	10	1480	2110	150	37	f.a.
SM14	10	400	870	330	335	f.a.
Mean ±	s.e.m.	970 ± 225	1590 ± 430	220 ± 50	110 ± 75	
B3	180	37	0.25	146	211	f.a.
C3	180	81	0.24	51	*	f.a.
B4	180	66	0.34	123	*	f.a.
SM16	180	2.3	f.a.	5.2	236	f.a.
SM21	180	f.a.	*	*	*	*
SM22	180	f.a.	*	*	*	*
Mean ±	s.e.m.	46 ± 17	0.28[a] ± 0.03	81 ± 32	224 ± 12[b]	
C1	360	53	0.44	5.4	12	f.a.
C2	360	38	0.34	6.6	18	f.a.
A3	360	27	0.47	4.4	10	f.a.
A2	360	47	0.57	0.22	235	f.a.
Mean ±	s.e.m.	41 ± 6	0.46 ± 0.05	4.2 ± 1.4	69 ± 55	

n.d. = not detectable (< 125 pg/g)
f.a. = failed analysis
* = not sampled
[a] n=3
[b] n=2

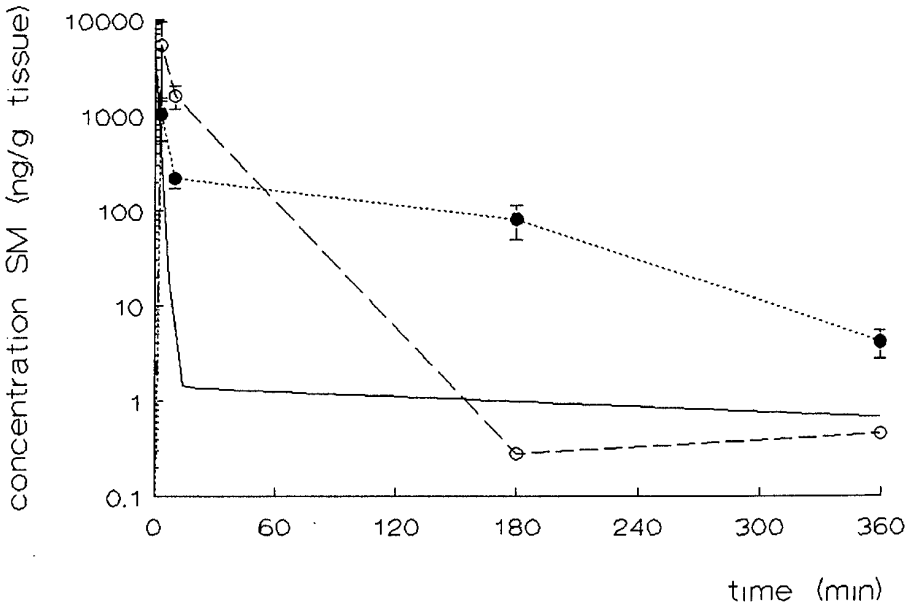

Figure 12. Concentration (ng/g, ± s.e.m.) of intact SM in lung (○) and spleen (●) of hairless guinea pigs at 3, 10, 180 and 360 min after i.v. administration of 8.2 mg/kg, which corresponds with 1 LD50 (96-h). The solid line represents the concentration-time course of SM in blood (ng/ml).

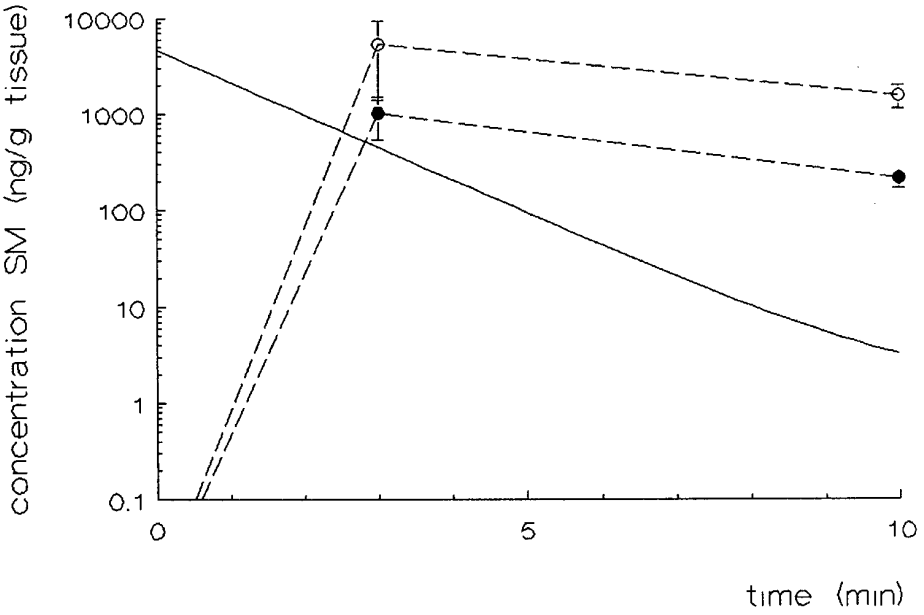

Figure 12a. The first 10 min of Figure 12 on an expanded scale.

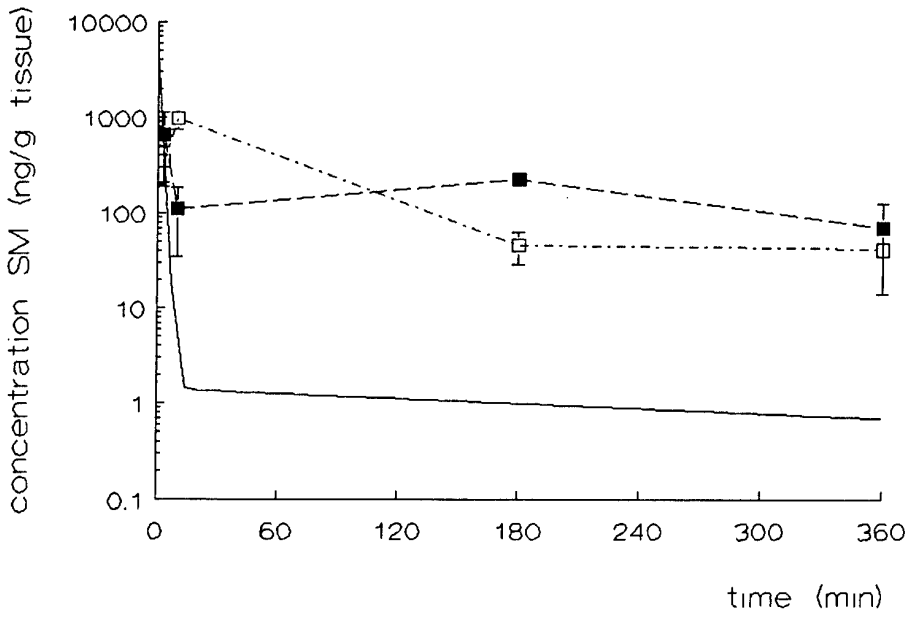

Figure 13. Concentration (ng/g, ± s.e.m.) of intact SM in liver (❑) and bone marrow (■) of hairless guinea pigs at 3, 10, 180 and 360 min after i.v. administration of 8.2 mg/kg, which corresponds with 1 LD50 (96-h). The solid line represents the concentration-time course of SM in blood (ng/ml).

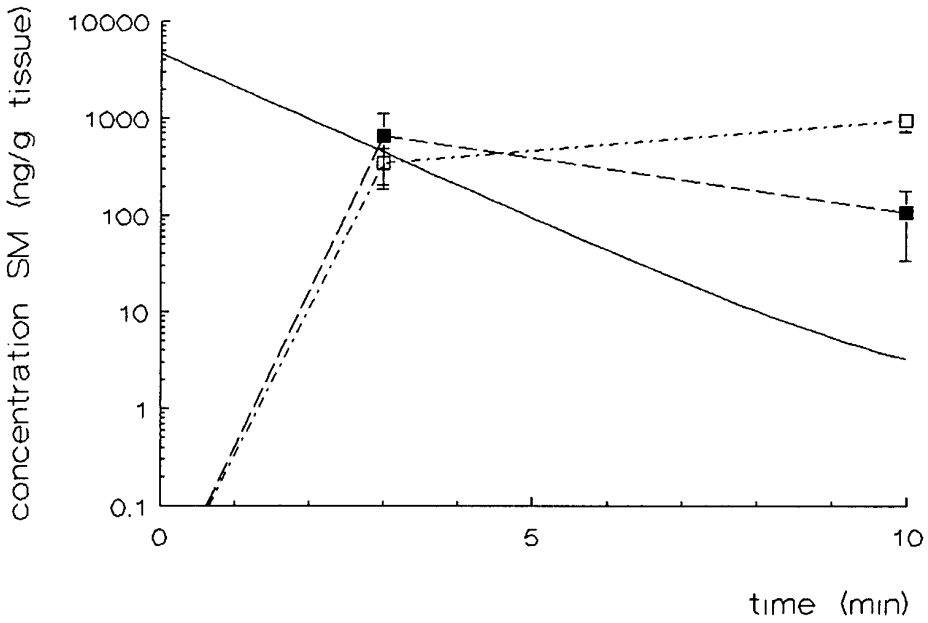

Figure 13a. The first 10 min of Figure 13 on an expanded scale.

III.4.c. Toxicokinetics of the major adduct of sulfur mustard to DNA

From the hairless guinea pigs receiving 1 LD50 SM (i.v., 8.2 mg/kg) in the toxicokinetic experiments, tissues were sampled at 3 time points after administration, i.e., at 180, 300, and 360 min after administration. Separate experiments were performed in which the samples were taken at 3, 10, 1440 and 2880 min after administration. The tissue samples were obtained from blood, liver, lung, spleen, bone marrow and small intestine. In these tissues the concentration of the major adduct of SM to DNA, i.e., 7-SM-gua, was determined via ISB. The concentration was expressed as number of adducts per 10^7 nucleotides. The data are presented in Table 10. It is clear from these data that 7-SM-gua can be detected in all selected tissues after intravenous administration of SM at a dose corresponding with 1 LD50. The results are also presented in Figures 14-16. For reasons of comparison, the results for blood are shown in each of these Figures.

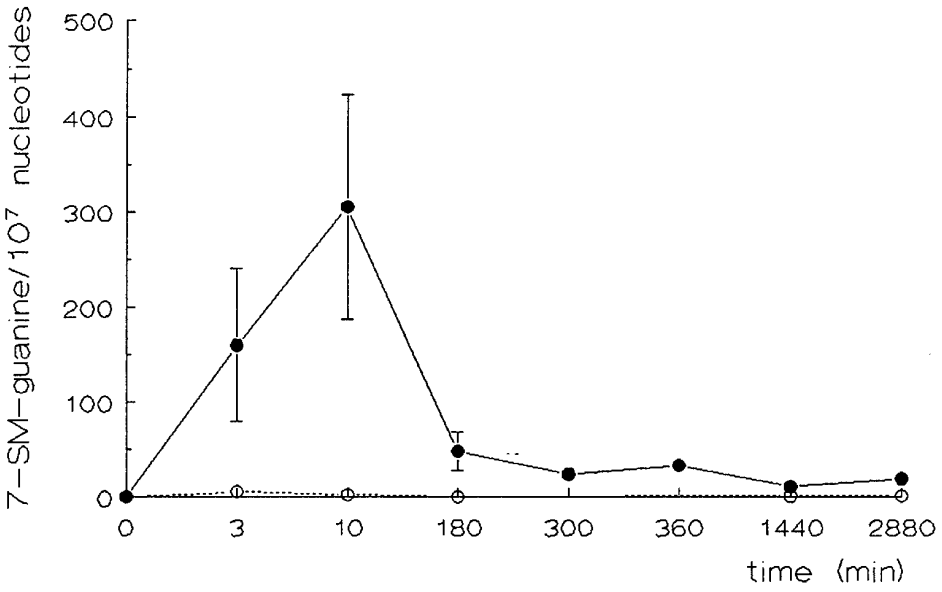

Figure 14. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± s.e.m., in blood (○) and lung (●) of hairless guinea pigs at various time points after intravenous administration of 8.2 mg SM per kg, which corresponds with 1 LD50 (96-h). The labels along the X-axis respresent the time points (in min) after administration.

Table 10. Concentration (number/10^7 nucleotides) of 7-SM-gua in various tissues at 3, 10, 180, 300, 360, 1440 and 2880 min after intravenous administration of sulfur mustard to anesthetized hairless guinea pigs at a dose of 8.2 mg/kg, which corresponds with 1 LD50 (96-h). Mean values with s.e.m. (n=4, unless stated otherwise) are also presented.

Animal #	Time (min)	Concentration 7-SM-gua in tissue (number of adducts per 10^7 nucleotides)					
		Blood	Liver	Lung	Spleen	Bone marrow	Small intestine
SM1	3	11.4	2.4	387	n.d.	0.1	1.6
SM2	3	0.2	2.7	106	n.d.	3.7	2.5
SM5	3	4.7	0.1	127	27	0.3	4
SM13	3	§	2.9	27	51	0.13	28
Mean ±	s.e.m.	5.4 ± 3.2[a]	2.0 ± 0.6	160 ± 80	20 ± 12	1.0 ± 0.9	9 ± 6
SM3	10	1.1	0.3	129	49	48	26
SM4	10	5.4	2	543	3.9	5	15
SM6	10	n.d.	2.6	473	0.2	3	2.6
SM14	10	§	0.8	74	38	3.1	15.5
Mean ±	s.e.m.	2.2 ± 1.6[a]	1.4 ± 0.5	305 ± 118	23 ± 12	15 ± 11	15 ± 5
B3	180	*	12	100	44	19	*
B4	180	*	5	55	47	8.7	0.2
C3	180	*	4	28	0	*	0
SM16	180	0.3	0.05	8.2	3	1.7	1.6
Mean ±	s.e.m.	0.3[b]	5.3 ± 2.5	48 ± 20	24 ± 13	10 ± 5[a]	0.6 ± 0.5[a]
B1	300	*	9	33	18	2.3	n.d.
B2	300	*	5	14	9	6.7	n.d.
D1	300	*	0.8	15	10	6	n.d.
D2	300	*	0.6	34	7	19	n.d.
Mean ±	s.e.m.	*	3.0 ± 1.6	24 ± 6	11 ± 2	8 ± 4	n.d.
A1	360	*	*	99	34.5	12	n.d.
A2	360	*	0.3	19	2	11	n.d.
A3	360	*	5	44	11.4	6	n.d.
A4	360	*	5	18	10.3	10.7	4.6
A5	360	*	5	28	4	26	0.2.
B5	360	*	1	39	7	1	9
C1	360	*	2	5.8	3.9	2	n.d.
C2	360	*	0.5	25	9.2	6	0.3
D3	360	*	4	22	19	8	n.d.
Mean ±	s.e.m.	*	2.8 ± 0.8[c]	33 ± 9[d]	11 ± 3[d]	9.2 ± 2.5[d]	1.6 ± 1.1[d]
?	1440	*	n.d.	9	2	1	2
?	1440	*	n.d.	5	1	0.3	1
SM7	1440	0.9	0.4	18	2.1	n.d.	4
Mean ±	s.e.m.	0.9[b]	0.1 ± 0.1[a]	11 ± 4[a]	1.7 ± 0.4[a]	0.4 ± 0.3[a]	2.3 ± 0.9[a]
SM8	2880	1.2	0.2	31	0.5	0.5	3.5
?	2880	*	n.d.	22	2.5	0.3	2
?	2880	*	n.d.	4	0.5	n.d.	n.d.
		1.2[b]	0.07 ± 0.07[a]	19 ± 8[a]	1.2 ± 0.7[a]	0.3 ± 0.2[a]	1.8 ± 1.0[a]

§ DNA could not be isolated from the sample; * not sampled; [a] n=3; [b] n=1; [c] n=8; [d] n=9;

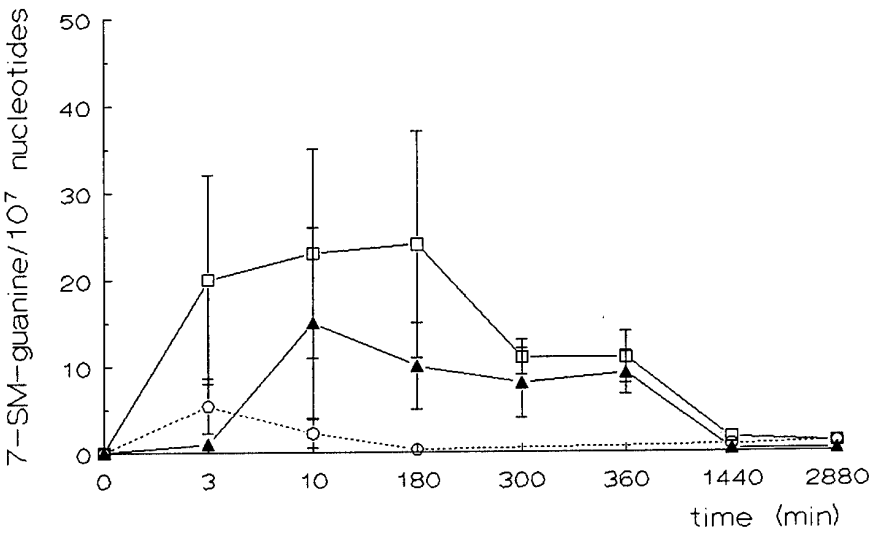

Figure 15. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± s.e.m., in blood (○) spleen (❑) and bone marrow (▲) of hairless guinea pigs at various time points after intravenous administration of 8.2 mg SM per kg, which corresponds with 1 LD50 (96-h). The labels along the X-axis respresent the time points (in min) after administration.

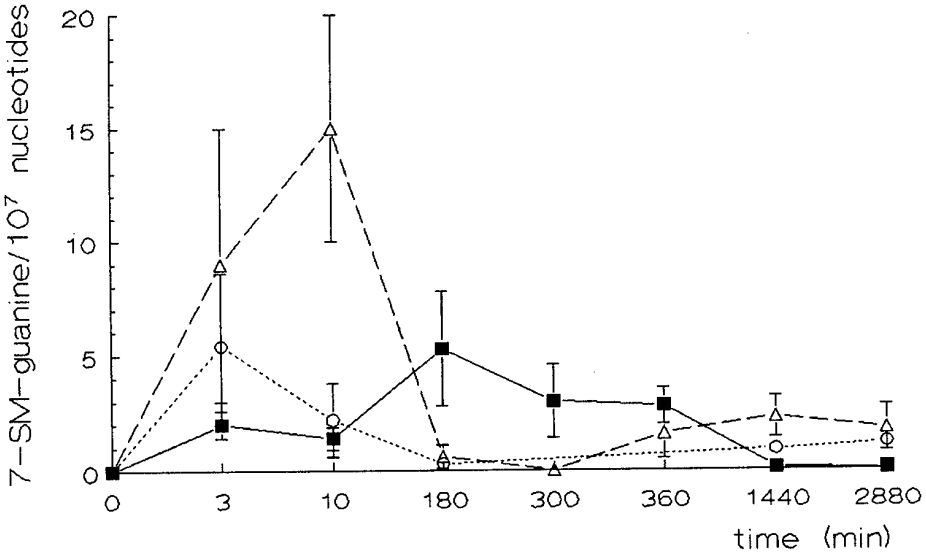

Figure 16. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± s.e.m., in blood (○), liver(■) and small intestine (Δ) of hairless guinea pigs at various time points after intravenous administration of 8.2 mg SM per kg, which corresponds with 1 LD50 (96-h). The labels along the X-axis respresent the time points (in min) after administration.

III.5 TOXICOKINETICS OF SULFUR MUSTARD IN ANESTHETIZED HAIRLESS GUINEA PIGS AFTER INTRAVENOUS BOLUS ADMINISTRATION OF A DOSE CORRESPONDING WITH 0.3 LD50

III.5.a. Toxicokinetics of sulfur mustard in blood

Male hairless guinea pigs were anesthetized, the jugular vein was made accessible, and a carotid canulla was inserted. SM was injected into the jugular vein in a dose of 2.46 mg/kg, which corresponds with 0.3 LD50 (96-h). Blood samples were drawn via the carotid canulla just before administration of the toxicant (time 0) and at 0.5, 1, 2, 4, 10, 15, 20, 30, 40, 60, 90, 120, 180, and 240 min after administration. The size of the samples ranged from 0.1 ml in the first samples up to 3 ml for the final two sampling times. In order to avoid too much strain on the individual animals, the sampling times were divided between two animals. Theoretically, 12 animals would be needed to obtain 6 values for each time point. However, since for some of the samples the analysis failed, additional experiments had to be performed. A total number of 16 hairless guinea pigs was used to complete this task. For time point 60 min only five values are available. It was considered to be inefficient to use an additional animal only for the purpose of obtaining one blood sample. In our experience, there is usually not much difference between mean values for n=5 or n=6.

The blood samples were extracted with ethyl acetate to which the internal standard was added. The extracts were analyzed with GLC configuration 4 (Cf. Section II.2)`.

The mean concentrations with standard error of the mean are presented in Table 11, whereas the blood concentrations of SM measured in the individual animals are presented in Table 12. The mean concentration-time course is shown in Figure 17. Toxicokinetic paremeters derived from the concentration-time course are presented in Table 13.

Table 11. Mean concentration in blood (ng/ml ± s.e.m.) of SM in anesthetized hairless guinea pigs at various time points after i.v. administration of 0.3 LD50 (2.46 mg/kg) of SM.

Time (min)	Concentration of SM (ng/ml blood ± s.e.m.)		
0	n.d.		(n=14)
0.5	574	± 116	(n=6)
1	304	± 122	(n=7)
2	146	± 85	(n=8)
4	27	± 21	(n=7)
10	8.3	± 2.8	(n=6)
15	2.4	± 1.1	(n=6)
20	1.6	± 0.7	(n=6)
30	1.3	± 0.5	(n=6)
40	1.6	± 0.5	(n=6)
60	1.6	± 0.8	(n=5)
90	1.0	± 0.5	(n=7)
120	1.1	± 0.6	(n=6)
180	1.1	± 0.9	(n=6)
240	0.4	± 0.4	(n=6)

n.d. = not detectable (< 5 pg/ml)

The blood concentration-time profile of SM after intravenous administration of a dose corresponding with 0.3 LD50 resembles the profile observed for 1 LD50. For both doses a clearcut biphasic profile was observed, with a very rapid first (distribution) phase and a slow second (elimination) phase.

Table 12. Concentrations in blood of SM (ng/ml) in individual anesthetized male hairless guinea pigs at various time points after intravenous administration of a dose of 2.46 mg/kg, which corresponds with 0.3 LD50 (96-h).

	Concentration in blood of SM (ng/ml) Hairless guinea pig #															
Time (min)	G1 (628)[a]	H1 (648)[a]	E2 (715)[a]	F2 (819)[a]	G2 (832)[a]	H2 (1001)[a]	E3 (951)[a]	F3 (955)[a]	G3 (912)[a]	H3 (799)[a]	E4 (737)[a]	F4 (759)[a]	G4 (912)[a]	H4 (799)[a]	E5 (797)[a]	F5 (940)[a]
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0.5	f.a.	*	490	*	808	*	250	*	251	*	777	*	871	*	*	*
1	*	374	*	331	*	288	*	n.d.	*	958	56.7	*	*	123	*	*
2	*	19.9	729	*	*	14.8	115	*	*	35.8	*	168	74.7	12.9	*	*
4	8.8	*	*	7.9	8.9	*	*	151	5.2	*	*	5.8	4.1	*	*	*
10	*	4.5	19.2	*	7.7	*	13.5	*	n.d.	*	4.8	*	*	*	*	*
15	*	n.d.	n.d.	*	*	3.7	*	n.d.	*	f.a.	*	5.3	*	5.5	*	*
20	*	n.d.	*	*	*	3.4	n.d.	*	*	f.a.	3.1	*	3.0	n.d.	n.d.	*
30	f.a.	*	*	f.a.	2.7	*	*	n.d.	2.5	*	*	2.6	*	*	n.d.	0.6
40	n.d.	*	*	*	3.0	*	n.d.	*	1.8	*	2.3	*	2.1	n.d.	*	*
60	n.d.	*	*	1.6	2.5	*	*	n.d.	f.a.	*	*	4.1	*	*	*	*
90	*	n.d.	*	*	*	2.3	n.d.	*	*	f.a.	2.0	f.a.	2.5	n.d.	n.d.	n.d.
120	*	n.d.	*	3.4	*	0.7	*	n.d.	*	f.a.	*	2.4	*	n.d.	*	*
180	n.d.	*	*	*	4.5	*	n.d.	*	f.a.	*	2.3	*	n.d.	*	0.7	n.d.
240	n.d.	*	*	*	n.d.	*	n.d.	*	f.a.	*	2.2	*	n.d.	*	n.d.	n.d.

[a] Weight in grams

n.d. = not detectable (< 5 pg/ml)

f.a. = failed analysis

* = not determined

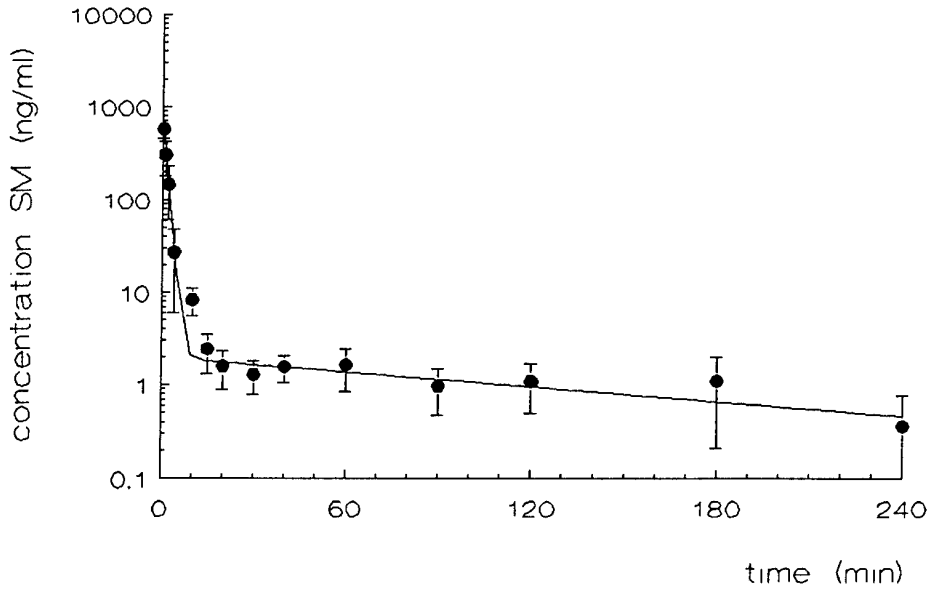

Figure 17. Mean concentration-time course of SM in the blood of anesthetized hairless guinea pigs after i.v. administration of 2.46 mg SM/kg, which corresponds with 0.3 LD50 (96-h).

Table 13. Toxicokinetic parameters[a] of SM in anesthetized hairless guinea pigs after intravenous administration of 2.46 mg/kg, which corresponds with 0.3 LD50 (96-h).

Parameter	0.3 LD50
Number of exponents	2
F-ratio	-1.6
A ($ng.ml^{-1}$)	826
B ($ng.ml^{-1}$)	1.97
a (min^{-1})	0.85
b (min^{-1})	0.0061
AUC ($ng.min.ml^{-1}$)	1294
C_0 ($ng.ml^{-1}$)	828
$k_{1,2}$ (min^{-1})	0.209
k_{el} (min^{-1})	0.64
$k_{2,1}$ (min^{-1})	0.0081
$t_{½,dis}$ (min)	0.81
$t_{½,el}$ (min)	114
V_1 ($l.kg^{-1}$)	2.97
V_{dss} ($l.kg^{-1}$)	79.55
Cl ($l.min^{-1}.kg^{-1}$)	1.90
MRT (min)	41.85

[a] The concentration of SM in blood at time t is described by: $[SM] = A*e^{-a*t} + B*e^{-b*t}$
Abbreviations used: AUC, area under the curve; C_0, retrapolated concentration in the central compartment at time 0; $k_{1,2}$, rate constant of transfer from compartment 1 to compartment 2; k_{el}, rate constant of elimination; $k_{2,1}$, rate constant of transfer from compartment 2 to compartment 1; $t_{½,dis}$, distribution half-life; $t_{½,el}$, terminal half-life; V_1, volume of the central compartment; V_{dss}, volume of distribution under steady-state; Cl, total body clearance; MRT, mean residence time.
[b] Critical value $F_{2,10} = 4.10$ (p=0.05)

III.5.b. Distribution of intact sulfur mustard in tissues

Tissue concentrations of intact SM were measured in various tissues at three time-points after intravenous administration of a dose of SM corresponding with 0.3 LD50. The tissues were liver, lung, spleen, bone marrow and abdominal fat. The time-points were 10 min after intravenous administration of SM, and halfway and at the end of the toxicokinetic experiment, i.e., 120 and 240 min after administration, respectively. The extracts of homogenized tissues in ethyl acetate were analyzed with GLC configuration 6 (*cf.* Section II.2). The results are presented in Table 14 as well as Figures 18 and 19.

Table 14. Concentration (ng/g) of intact SM in various tissues sampled at 3, 10, 180 or 240 min after intravenous administration of 2.5 mg/kg SM to anesthetized hairless guinea pigs, which corresponds with 0.3 LD50 (96-h). Mean values with s.e.m. (n=4, unless stated otherwise) are also presented.

Animal #	Time (min)	Concentration SM in tissue (ng/g)				
		Liver	Lung	Spleen	Marrow	Fat
SM-1	10	43	3.4	9.6	7.8	f.a.
SM-2	10	38	f.a.	f.a.	f.a.	f.a.
SM-3	10	93	f.a.	f.a.	f.a.	f.a.
SM-4	10	12	f.a.	1.0	35	f.a.
Mean ±	s.e.m.	46 ± 17	3.4[c]	5 ± 4[b]	22 ± 14[b]	
F4	120	0.37	0.54	0.22	n.d.	f.a.
H3	120	f.a.	f.a.	f.a.	3.8	f.a.
H4	120	f.a.	f.a.	f.a.	f.a.	f.a.
F1	120	6.1	f.a.	f.a.	f.a.	f.a.
Mean ±	s.e.m.	3 ± 3[b]	0.54[c]	0.22[c]	1.9 ± 1.9[b]	
E4	240	0.52	0.97	n.d.	n.d.	f.a.
G3	240	1.6	f.a.	f.a.	f.a.	f.a.
G4	240	4.1	f.a.	f.a.	f.a.	f.a.
F2	240	f.a.	0.3	f.a.	n.d.	f.a.
Mean ±	s.e.m.	2.1 ± 1.1[a]	0.6 ± 0.3[b]	n.d.[c]	n.d.[b]	

n.d. = not detectable (< 125 pg/g)
f.a. = failed analysis
* = not sampled
[a] n=3
[b] n=2
[c] n=1

At 10 min after administration, the concentrations of SM in liver, lung, spleen and bone marrow exceed that in blood. However, at 120 min after administration, only the concentrations intact SM in liver and bone marrow exceed those in blood, whereas at 240 min this is only the case for liver. A substantial number of analyses have failed, due to technical problems with the gas chromatograph. Unfortunately, for most of these failed samples we did not have sufficient material to repeat the analysis.

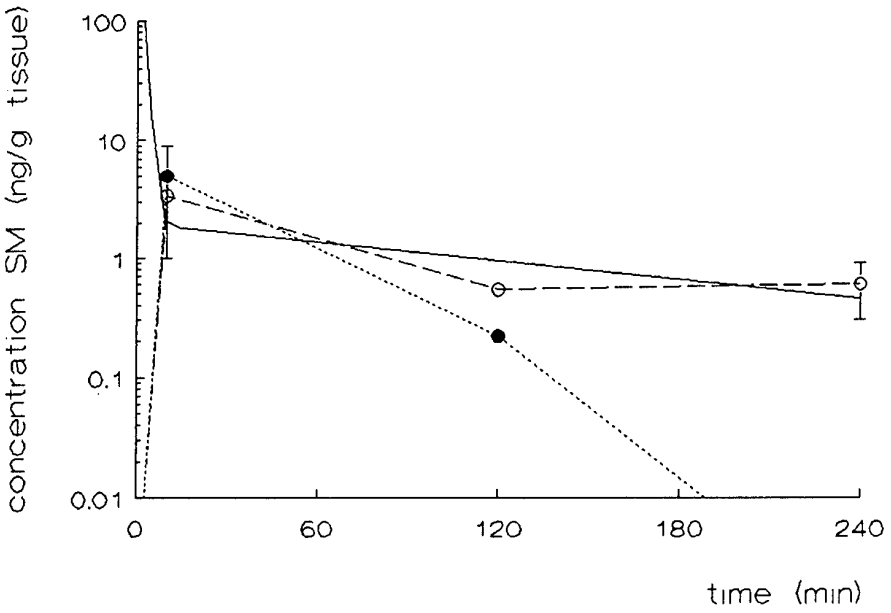

Figure 18. Concentration (ng/g, ± s.e.m.) of intact SM in lung (○) and spleen (●) of hairless guinea pigs at 10, 120 and 240 min after i.v. administration of 2.46 mg/kg, which corresponds with 0.3 LD50 (96-h). The solid line represents the concentration-time course of SM in blood (ng/ml).

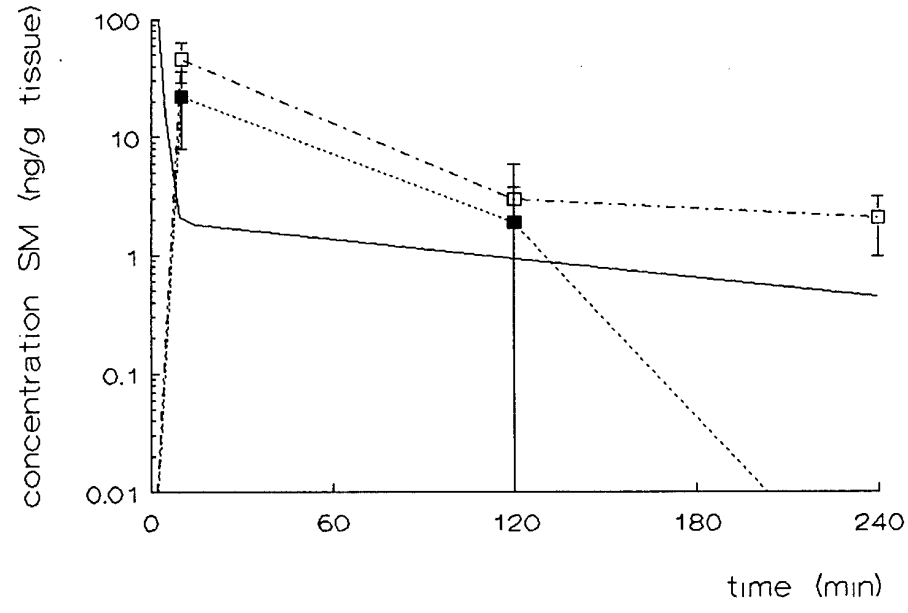

Figure 19. Concentration (ng/g, ± s.e.m.) of intact SM in liver (❑) and bone marrow (■) of hairless guinea pigs at 10, 120 and 240 min after i.v. administration of 2.46 mg/kg, which corresponds with 0.3 LD50 (96-h). The solid line represents the concentration-time course of SM in blood (ng/ml).

III.5.c. Toxicokinetics of the major adduct of sulfur mustard to DNA

From the hairless guinea pigs receiving 0.3 LD50 SM (i.v., 2.46 mg/kg) in the toxicokinetic experiments, tissues were sampled at 2 time points after administration, i.e., at 2 and 4 h after administration. Separate experiments were performed in which samples were taken at 10 min and 48 h after administration. The tissues sampled were blood, liver, lung, spleen, bone marrow and small intestine. In these tissues the concentration of the major adduct of SM to DNA, i.e., 7-SM-gua, was determined via ISB. The concentration was expressed as number of adducts per 10^7 nucleotides. The results are presented in Table 15.

Table 15. Concentration (number of adducts/10^7 nucleotides) of 7-SM-gua in various tissues at 10, 120, 240 and 2880 min after intravenous administration of sulfur mustard to anesthetized hairless guinea pigs at a dose of 2.46 mg/kg, which corresponds with 0.3 LD50 (96-h). Mean values with s.e.m. (n=4, unless stated otherwise) are also presented.

Animal #	Time (min)	Concentration 7-SM-gua in tissue (number of adducts per 10^7 nucleotides)					
		Blood	Liver	Lung	Spleen	Bone marrow	Small intestine
SM-1	10	9.3	n.d.	2.6	1	n.d.	n.d.
SM-2	10	2.9	n.d.	2.9	0.2	n.d.	n.d.
SM-3	10	3.9	n.d.	4.7	1	n.d.	1.1
SM-4	10	§	n.d.	3.3	0.3	n.d.	0.2
Mean ±	s.e.m.	5 ± 2[a]	n.d.	3.4 ± 0.5	0.6 ± 0.2	n.d.	0.3 ± 0.3
H1	120	n.d.	n.d.	3.7	2.3	0.1	1.2
F3	120	n.d.	n.d.	0.06	n.d.	n.d.	0.05
H2	120	n.d.	n.d.	0.4	0.2	n.d.	n.d.
F4	120	0.05	n.d.	0.15	0.06	n.d.	0.05
H3	120	0.05	n.d.	0.2	0.3	n.d.	n.d.
H4	120	n.d.	n.d.	0.4	0.05	n.d.	n.d.
F1	120	§	n.d.	*	0.2	0.1	0.07
Mean ±	s.e.m.	0.02 ± 0.01[b]	n.d.[c]	0.8 ± 0.6[b]	0.4 ± 0.3[c]	0.03 ± 0.02[c]	0.2 ± 0.2[c]
G1	240	n.d.	n.d.	0.8	0.5	0.05	0.05
F2	240	n.d.	n.d.	0.05	0.05	n.d.	0.9
E3	240	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
G2	240	n.d.	n.d.	0.05	n.d.	n.d.	n.d.
E4	240	n.d.	n.d.	1.4	0.3	n.d.	0.3
G3	240	n.d.	n.d.	0.7	0.35	n.d.	n.d.
G4	240	0.05	n.d.	0.05	n.d.	n.d.	n.d.
Mean ±	s.e.m.	0.01 ± 0.01[c]	n.d.[c]	0.4 ± 0.2[c]	0.2 ± 0.1[c]	0.01 ± 0.01[c]	0.2 ± 0.1[c]
SM-5	2880	1.7	n.d.	1.2	n.d.	n.d.	n.d.
SM-6	2880	0.1	n.d.	0.5	n.d.	n.d.	n.d.
SM-7	2880	0.7	n.d.	0.3	n.d.	n.d.	n.d.
SM-8	2880	6.2	n.d.	0.4	n.d.	n.d.	n.d.
		2.2 ± 1.4	n.d.	0.6 ± 0.2	n.d.	n.d.	n.d.

n.d. = not detectable

§ DNA could not be isolated from the sample

[a] n=3

[b] n=6

[c] n=7

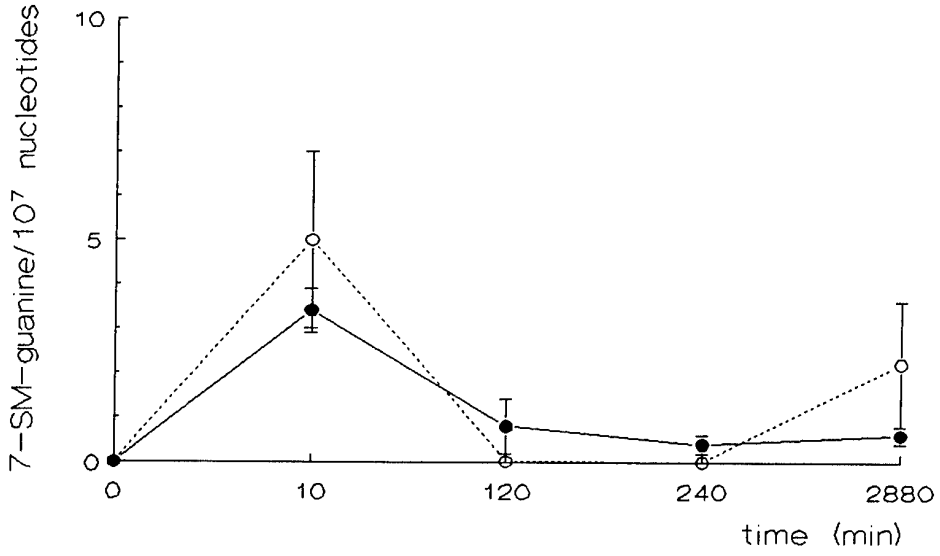

Figure 20. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± s.e.m., in blood (○) and lung (●) of hairless guinea pigs at various time points after intravenous administration of 2.46 mg SM per kg, which corresponds with 0.3 LD50 (96-h). The labels along the X-axis represent the time points (in min) after administration.

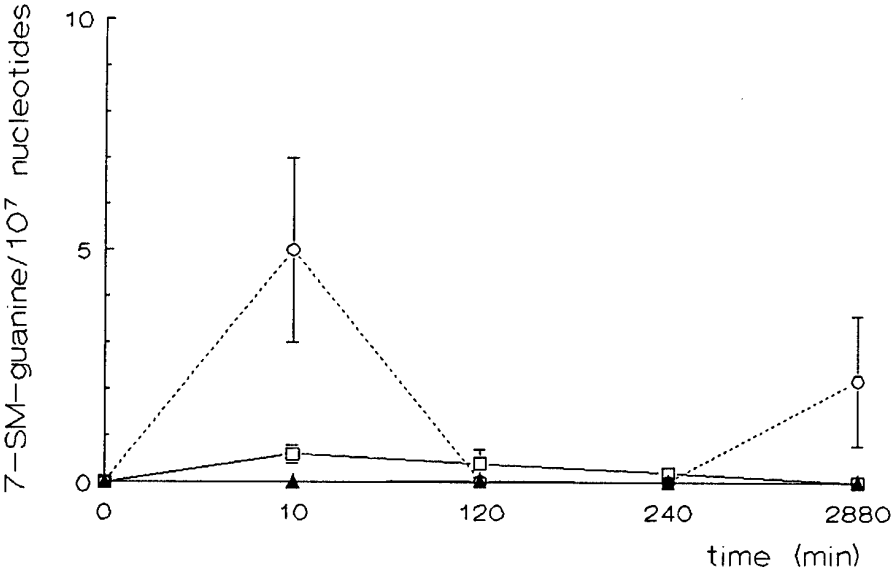

Figure 21. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± s.e.m., in blood (○), spleen (□) and bone marrow (▲) of hairless guinea pigs at various time points after intravenous administration of 2.46 mg SM per kg, which corresponds with 0.3 LD50 (96-h). The labels along the X-axis represent the time points (in min) after administration.

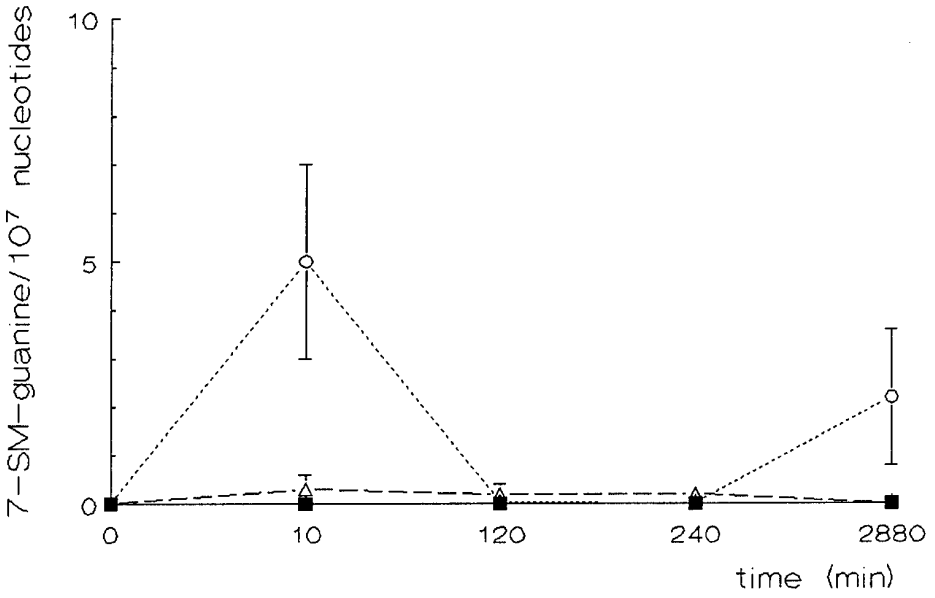

Figure 22. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± s.e.m., in blood (○), liver (■) and small intestine (Δ) of hairless guinea pigs at various time points after intravenous administration of 2.46 mg SM per kg, which corresponds with 0.3 LD50 (96-h). The labels along the X-axis represent the time points (in min) after administration.

At 10 min after administration of a dose of SM corresponding with 0.3 LD50 DNA adducts could be measured in blood, lung, spleen and small intestine, albeit at rather low concentrations. The highest concentrations were observed in blood and lung. No adducts could be detected at this time point in liver and bone marrow. In the liver adducts could not be detected in any of the time points, whereas in bone marrow very low concentrations could be measured at 120 and 240 min after administration. In blood and liver the concentrations of 7-SM-gua decreased considerably in the time period between 10 and 120 min. Interestingly, the adduct concentration in blood increased in the time period between 240 and 2880 min, for which we have no explanation at this point.

III.6 APPARATUS FOR NOSE-ONLY EXPOSURE OF HAIRLESS GUINEA PIGS TO SULFUR MUSTARD VAPOR

The apparatus designed within the context of Cooperative Agreement DAMD17-90-Z-0034 for respiratory exposure of guinea pigs to soman and sarin vapor (Benschop and Van Helden, 1993) was modified in order to meet the requirements of the current study.
Firstly, the internal volume of the vapor generation apparatus was reduced, in order to shorten the response time when increasing or decreasing the vapor concentration. Secondly, the position where the airstream was sampled for gas chromatographic determination of the concentration of the agent, was moved closer to the point were the animal is positioned. Furthermore, the valve through which the toxicant vapor passes, which was made of synthetic material, was replaced with a heated valve (40 °C) with a brass interior.
The critical orifices which were positioned in various tubings in the previous study were omitted for the purpose of this study. Furthermore, the 'underpressure chamber' around the modified Battelle tube was removed. Since the exposure chamber was placed in a perspex cabinet with air suction, there was no need for the 'underpressure chamber'.
The most significant modification of the apparatus is the replacement of the PTFE front chamber from which the animal breathes with a stainless steel front chamber. PTFE is known to adsorb SM, whereas stainless steel is relatively inert towards this agent. Before using stainless steel we tried a glass front chamber, but this proved to be too vulnerable, and was therefore considered to be unacceptable from a safety point of view.

Due to the lower volatility of SM (900 $mg.m^{-3}$ at 25 °C) in comparison with soman and sarin (3,900 and 17,000 $mg.m^{-3}$ at 25 °C, respectively) (FOA, 1992) only low vapor concentrations of *ca.* 50 $mg.m^{-3}$ could be reached under normal operating conditions. Theoretically, the highest vapor concentration that can be reached at room temperature (20 °C) is *ca.* 600 $mg.m^{-3}$, which already indicates that it will very difficult to generate SM vapor in air with a concentration of 300
$mg.m^{-3}$ in a dynamic system, in particular at 50 % relative humidity. One option would be to mix dry air saturated with SM vapor with air satured with water vapor. A second option would be to lead air with a relative humidity of 50 % through neat agent. Certainly the second option would be more promising, although one can anticipate some degradation of SM in the generation vial. When SM evaporates by leading a stream of air through the neat agent, the temperature decreases, which further reduces the evaporation of the agent. This explains why we were unable to generate concentrations higher than 50 $mg.m^{-3}$. Straightforward thermostatting of the generation vial would not solve the problem since condensation would occur in any cold spot present in the generation apparatus. The only adequate solution to this problem is thermostatting the generation apparatus as a whole. A glass-blower rebuilt the generation apparatus with double-walled glass tubes. The system was thermostatted with water at the desired temperature (35-40 °C). The new configuration was approved with respect to safety by a group of experts from TNO Prins Maurits Laboratory. With this set-up we were able to continuously generate SM vapor in air at concentrations up to at least 350 $mg.m^{-3}$ and a relative humidity of 50 %.

Schematic representations of the generation apparatus and the exposure chamber are shown in Figures 23a and b. Figure 24 is a photograph of the exposure module.

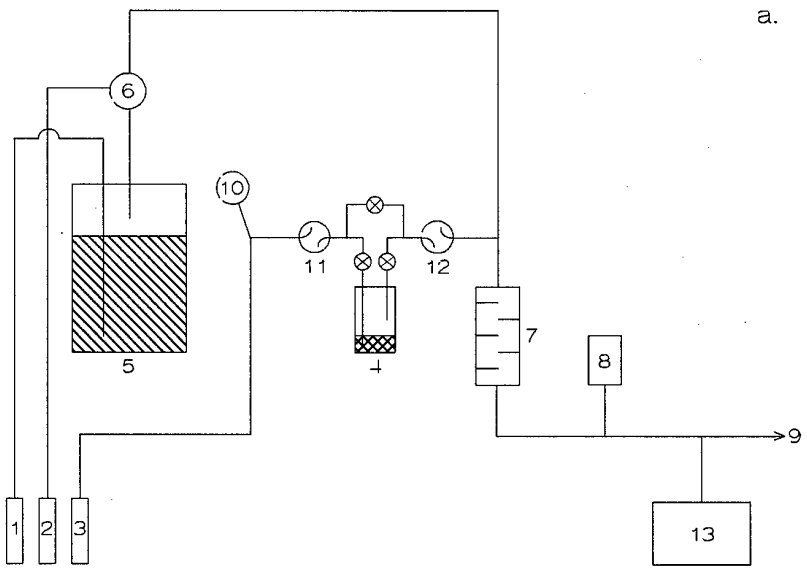

Figure 23a. Apparatus constructed for generation of sulfur mustard vapor. (1), (2) and (3) mass flow controllers; (4) vial containing sulfur mustard; (5) thermostatted water bath; (6) and (7) mixing chambers; (8) temperature/relative humidity meter; (9) towards the exposure module; (10) overpressure security; (11) and (12) splash heads; and (13) gas chromatograph with gas sampling valve.

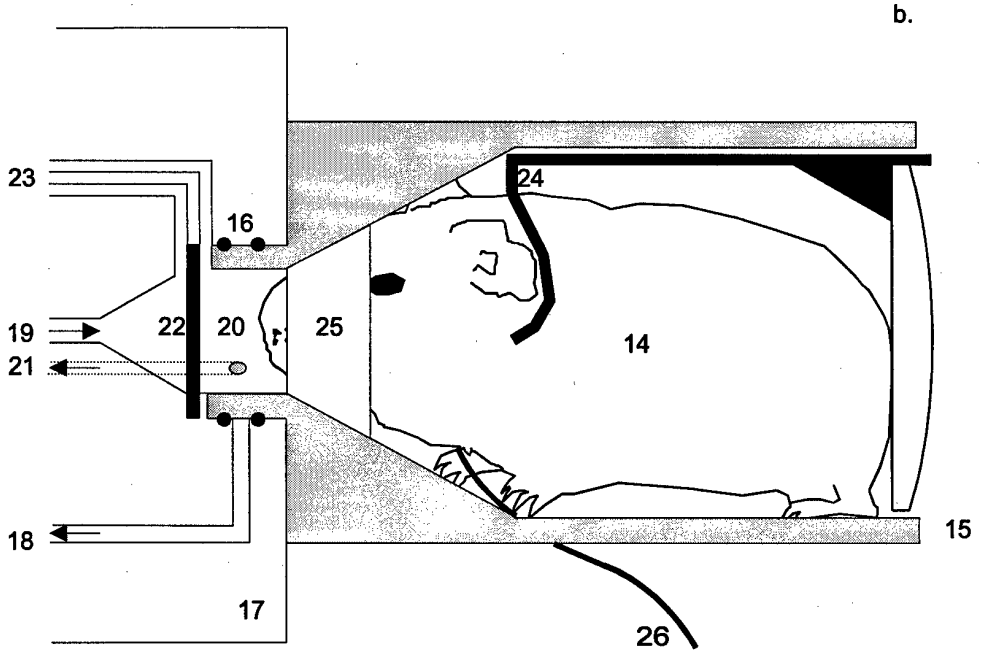

Figure 23b. Guinea pig (14) positioned in the modified Battelle tube (15). (16) O-rings for gastight connection of the tube to the body of the exposure apparatus (17); (18) tubing connected to an underpressure check for gastight connection of the tube; (19) glass tubing through which the sulfur mustard vapor is transported to the exposure chamber; (20) front chamber of the modified Battelle tube, from which the animal breathes; (21) outlet tubing of the front chamber; connected to a device for adjusting the flow through the front chamber via two threeway valves; (22) wire mesh resistance; (23) differential pressure measuring device; (24) fork for positioning the animal, (25) rubber mask; (26) carotid artery cannula. Arrows indicate flow directions.

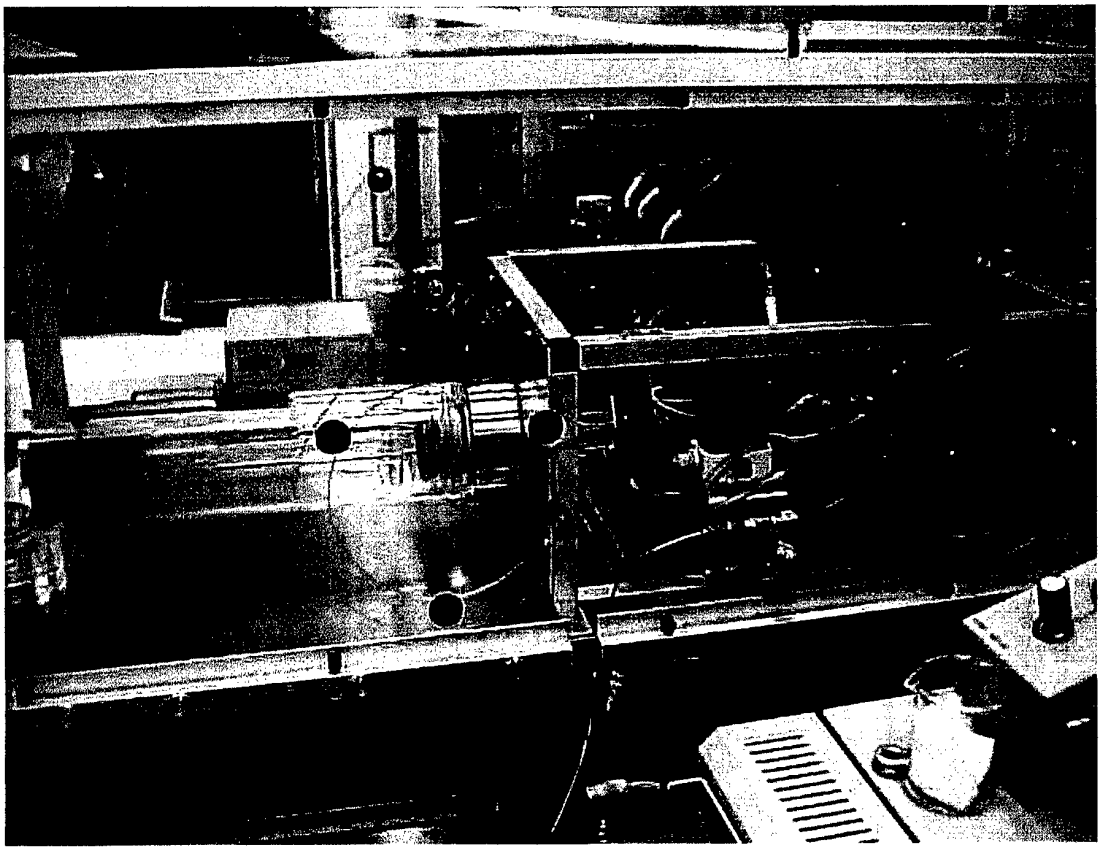

Figure 24. Photograph of the exposure module.

III.7 THE 96-h LCt50 OF SULFUR MUSTARD IN THE HAIRLESS GUINEA PIG FOR 5-MIN NOSE-ONLY EXPOSURE

Hardly any LC50 values for SM in species are documented. Vijayaraghavan (1994) reported a 96-h LC50 of 42.3 $mg.m^{-3}$ for mice. Unfortunately, the exposure time is not specified. Calvet *et al.* (1994) reported severe damage at 5 h and 14 days following an intratracheal injection of 0.3 mg/kg of SM in guinea pigs. For dose-range finding purposes we therefore started some pilot-experiments. Using the above-mentioned toxicity data as starting points, we calculated/estimated a first pilot dose of exposure as follows. Since in our proposed experiments animals would be exposed for 5 min to SM, 100 µg/guinea pig (350 g) must be taken up by the animal in order to induce clear damage according to Calvet *et al.* (1994). Since the tidal volume is 5 ml/kg, an animal of 350 g will inhale 1.7 ml per second. To induce damage, 20 µg/min should be inhaled (100 µg in 5 min), i.e., 20 µg.min/100 ml (1.7 x 60)= 200 $\mu g.min.l^{-1}$ = 200 $mg.min.m^{-3}$. In the window between 42.3 and 200 $mg.min.m^{-3}$ we decided to expose for 5 min 1 or 2 animals to SM vapor concentrations of 75, 100, 125, 150 or 175 $mg.m^{-3}$. After exposure two animals were housed per cage with access to food and water *ad libitum*. Several times a day during 4 days after exposure the animals were inspected and times to death were recorded. The animals exposed to 75, 100 or 125 $mg.m^{-3}$ survived the 96-h period, wheras one of the two animals exposed to 150 $mg.m^{-3}$ died. The animal exposed to 175 $mg.m^{-3}$ died within 24 h. One animal was exposed to a Ct of 1,100 $mg.min.m^{-3}$ in 11 min, in order to estimate at which Ct-value mortality would be near 100 %.
The results of the pilot experiment indicated that the LC50 would be within the concentration range chosen.

Next, the 96-h LCt50 value was determined from the mortality rates after 5-min exposure of groups of 8 animals to 5 different SM vapor concentrations ranging between 100 and 200 $mg.m^{-3}$ via probit analysis. In probit analysis, mortality rates of 0 and 100 % need to be avoided as much as possible. Therefore, 100 $mg.m^{-3}$ was chosen as the lowest challenge concentration. The results obtained for all individual animals are presented in Table 16. The results obtained for the two animals exposed to an SM vapor concetration of 75 $mg.m^{-3}$ and the single animal exposed to a Ct of 1,100 $mg.min.m^{-3}$, were not used for probit analysis.

In Table 17 the mortality rates for the five exposed groups are presented. Using probit analysis of these mortality data, the 96-h LC50 of SM was calculated to be 160 $mg.m^{-3}$ for a 5-min nose-only exposure, which corresponds with a LCt50-value of 800 $mg.min.m^{-3}$. The corresponding 95-% confidence intervals are 140-184 $mg.m^{-3}$ and 700-920 $mg.min.m^{-3}$, respectively. The results of this analysis are presented in Table 18 and Figure 25. Since proper respiratory LCt50 values for SM cannot be found in literature, we cannot compare the LCt50 value which we found in the hairless guinea pig with a value for other relevant species.

Table 16. Individual data of the determination of the 96-h LCt50 of SM in hairless guinea pigs.

[SM] ($mg.m^{-3}$)	Exposure time (min)	Ct-value ($mg.min.m^{-3}$)	Animal number	Weight (g)	Exposure date	Date of death	Survival time (h)
75	5	375	HT03	734	10/10/96		> 96
75	5	375	HT10	650	11/10/96		> 96
100	5	500	HT01	700	09/10/96		> 96
100	5	500	HT04	709	10/10/96		> 96
100	5	500	HT09	625	11/10/96		> 96
100	5	500	HT11	540	16/10/96		> 96
100	5	500	HT12	593	16/10/96		> 96
100	5	500	HT13	496	16/10/96		> 96
100	5	500	HT14	574	16/10/96		> 96
100	5	500	HT19	604	28/10/96		> 96
125	5	625	HT05	692	11/10/96	12/10/96	32
125	5	625	HT15	569	16/10/96		> 96
125	5	625	HT16	529	16/10/96		> 96
125	5	625	HT17	640	16/10/96		>96
125	5	625	HT18	531	16/10/96		> 96
125	5	625	HT20	644	28/10/96		> 96
125	5	625	HT21	575	28/10/96		> 96
125	5	625	HT22	637	28/10/96		> 96
150	5	750	HT06	723	11/10/96	12/10/96	32
150	5	750	HT23	645	28/10/96	29/10/96	19
150	5	750	HT24	625	28/10/96	29/10/96	19
150	5	750	HT27	738	28/10/96		> 96
150	5	750	HT26	630	28/10/96		> 96
150	5	750	HT28	620	04/11/96		> 96
150	5	750	HT29	607	04/11/96	05/11/96	27
150	5	750	HT30	660	04/11/96	05/11/96	20
175	5	875	HT07	680	11/10/96	12/10/96	31
175	5	875	HT31	653	04/11/96	05/11/96	20
175	5	875	HT32	595	04/11/96	06/11/96	43
175	5	875	HT33	620	04/11/96		> 96
175	5	875	HT34	580	04/11/96		> 96
175	5	875	HT35	660	04/11/96	05/11/96	19
175	5	875	HT36	564	04/11/96	05/11/96	19
175	5	875	HT37	576	04/11/96		> 96
200	5	1000	HT08	732	11/10/96		> 96
200	5	1000	HT38	748	11/11/96	12/11/96	22
200	5	1000	HT39	687	11/11/96	12/11/96	27
200	5	1000	HT40	623	11/11/96	12/11/96	22
200	5	1000	HT41	581	11/11/96		> 96
200	5	1000	HT42	640	11/11/96	12/11/96	21
200	5	1000	HT43	530	11/11/96	12/11/96	21
200	5	1000	HT44	564	11/11/96	15/11/96	93
200	5	1000	HT45	540	11/11/96	12/11/96	21
100	11	1100	HT02	700	09/10/96	10/10/96	18

Table 17. Number and percentage of dead animals per dosing group, at 96 h after nose-only exposure of anesthetized male hairless guinea pigs to sulfur mustard vapor in air.

[SM] ($mg.m^{-3}$)	Exposure time (min)	Ct-value ($mg.min.m^{-3}$)	Number of dead animals at 96 h/total number	Percentage deaths
100	5	500	0/8	0
125	5	625	1/8	12.5
150	5	750	5/8	62.5
175	5	875	5/8	62.5
200	5	1000	7/9	88

Table 18. LC10, LC30, LC50, LC70 and LC90 (96-h) with 95-% confidence limits, for 5-min nose-only exposure of anesthetized male hairless guinea pigs to sulfur mustard vapor in air, calculated via probit analysis[a].

LC	[SM] ($mg.m^{-3}$)	95-% Confidence limits ($mg.m^{-3}$)
10	116	77-134
30	140	113-157
50	160	140-184
70	182	163-232
90	220	189-342

[a] Probit equation: probit = 9.18*log[SM] - 15

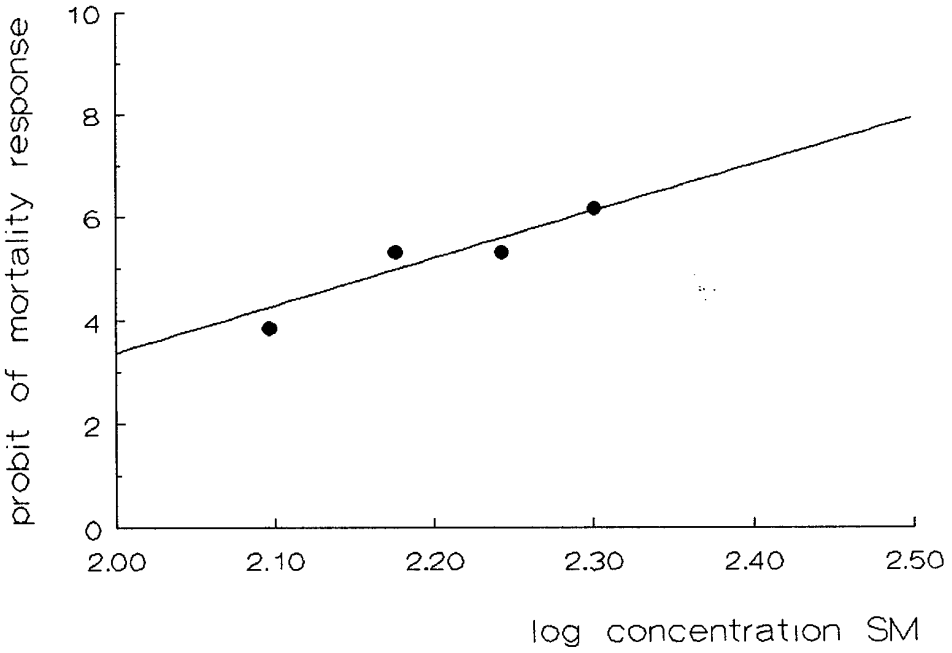

Figure 25. Probit of mortality of anesthetized male hairless guinea pigs nose-only exposed to sulfur mustard vapor in air for 5 min, versus the concentration of sulfur mustard vapor in air.

III.8 TOXICOKINETICS OF SULFUR MUSTARD AND ITS MAJOR DNA-ADDUCT IN ANESTHETIZED HAIRLESS GUINEA PIGS DURING AND AFTER 5-MIN NOSE-ONLY EXPOSURE TO 1 AND 0.3 LCt50

III.8.a. Toxicokinetics of sulfur mustard in blood

Anesthetized and restrained hairless guinea pigs were nose-only exposed to SM vapor in air with a concentration of 160 $mg.m^{-3}$ for 5 min. This yields a Ct-value of 800 $mg.min.m^{-3}$, which corresponds with 1 LCt50 (96-h). Blood samples were drawn via a carotid canulla just before exposure, and at 0.5, 1, 2, 3, 5, 10, 15, 20, 30, 40, 60, 90, 120, 180 and 240 min after the start of the exposure. The blood samples were analyzed with the TDAS-GC-MS configuration (configuration 4). In none of the blood samples SM could be detected (< 5 pg/ml), whereas the internal standard D_8-SM was easily detected. On the basis of these results, we decided to terminate this technical objective after 4 attempts. Since no SM could be detected in blood during and after 5-min nose-only exposure to 1 LCt50 SM, we considered exposure to 0.3 LCt50 to be futile. Instead, we tried to measure the toxicokinetics of SM in blood during and after nose-only exposure to 3 LCt50 SM. Such an exposure cannot be performed in 5 min, since this would require a SM vapor concentration of 480 $mg.m^{-3}$, which we cannot obtain with our generation setup. Therefore, it was decided to expose hairless guinea pigs nose-only for 8 min to SM vapor at a concentration of 300 $mg.m^{-3}$.

III.8.b. Distribution of intact sulfur mustard in tissues

Since SM could not be detected in the blood samples we decided not to try to measure SM in tissues.

III.8.c. Toxicokinetics of the major adduct of sulfur mustard to DNA

Tissues were sampled from anesthetized and restrained male hairless guinea pigs at 10, 115, 235 and 2880 min after ending a 5-min nose-only exposure to a concentration of SM vapor in air of 160 mg/m^3, yielding a Ct of 800 $mg.min.m^{-3}$ (1 LCt50). The tissues were blood, liver, lung, spleen, bone marrow and small intestine. DNA was isolated from tissues, after which the concentration of 7-SM-gua was determined with the immunoslotblot assay. The results are presented in Table 19. At 10 min after ending the exposure to 1 LCt50 7-SM-gua could only be measured in blood and lung. At later time points very low adduct levels were found occasionally in spleen, bone marrow and small intestine. The adduct level in blood appears to be relatively high, particularly in view of the fact that no intact SM could be detected in blood. At 115 min after ending the exposure the adduct level in blood has decreased *ca.* 10-fold, and remains stable up to 235 min. However, at 48 h (2880 min) after ending the exposure the mean 7-SM-gua concentration has increased fivefold, albeit with a large standard error of the mean. The adduct levels in the lung are consistently low. In liver, no adducts were found for these four time points. Tissues were also sampled from animals at 10 and 2880 min after ending a 5-min nose-only exposure to a concentration of SM vapor in air of 48 mg/m^3, yielding a Ct of 240 $mg.min.m^{-3}$ (0.3 LCt50). The tissues were blood, liver, lung, spleen, bone marrow and small intestine. DNA was isolated from tissues, after which the concentration of 7-SM-gua was determined with the immunoslotblot assay. The results are presented in Table 20. At 10 min after ending the nose-only exposure to 0.3 LCt50 7-SM-gua could be detected in blood and lung. The measured concentrations of 7-SM-gua are comparable to those measured at 10 min after exposure to 1 LCt50, which is rather surprising. At 48 h after ending the exposure the adduct levels have not significantly changed. No adducts were detectable in liver, spleen, bone marrow or small intestine.

Table 19. Concentration (number of adducts/10^7 nucleotides) of 7-SM-gua in various tissues at 10, 115, 235 and 2880 min after ending a 5-min nose-only exposure of restrained, anesthetized hairless guinea pigs to SM vapor in air at a concentration of 160 mg.m^{-3}, yielding a Ct-value of 800 mg.min.m^{-3} (1 LCt50 (96-h)). Mean values with s.e.m. (n=4, unless stated otherwise) are also presented.

Animal #	Time (min)	Concentration 7-SM-gua in tissue (number of adducts per 10^7 nucleotides)					
		Blood	Liver	Lung	Spleen	Bone marrow	Small intestine
HT50	10	1.3	n.d.	n.d.	n.d.	n.d.	n.d.
HT51	10	2.1	n.d.	0.3	n.d.	n.d.	n.d.
HT52	10	3.1	n.d.	0.4	n.d.	n.d.	n.d.
HT53	10	15	n.d.	2.3	n.d.	n.d.	n.d.
Mean ±	s.e.m.	5.4 ± 3.2	n.d.	0.8 ± 0.5	n.d.	n.d.	n.d.
M1	115	0.8	n.d.	n.d.	n.d.	n.d.	n.d.
K1	115	§	n.d.	n.d.	n.d.	n.d.	n.d.
K2	115	0.1	n.d.	n.d.	n.d.	0.01.	n.d.
Mean ±	s.e.m.	0.4 ± 0.4[a]	n.d.[a]	n.d.[a]	n.d.[a]	0.003 ± 0.003[a]	n.d.[a]
J1	235	0.3	n.d.	n.d.	0.01	0.06	n.d.
J2	235	0.4	n.d.	n.d.	n.d.	n.d.	n.d.
L1	235	1	n.d.	0.07	0.02	n.d.	0.03
Mean ±	s.e.m.	0.6 ± 0.2[a]	n.d.	0.02 ± 0.02[a]	0.01 ± 0.01[a]	0.02 ± 0.02[a]	0.01 ± 0.01[a]
HT46	2880	2.8	n.d.	0.4	n.d.	n.d.	n.d.
HT47	2880	0.2	n.d.	0.5	n.d.	n.d.	n.d.
HT48	2880	0.3	n.d.	0.5	n.d.	n.d.	n.d.
HT49	2880	6.1	n.d.	0.2	n.d.	n.d.	n.d.
		2.4 ± 1.4	n.d.	0.4 ± 0.1	n.d.	n.d.	n.d.

n.d. = not detectable
§ DNA could not be isolated from the sample
[a] n=3

Table 20. Concentration (number of adducts/10^7 nucleotides) of 7-SM-gua in various tissues at 10 and 2880 min after ending a 5-min nose-only exposure of restrained, anesthetized hairless guinea pigs to SM vapor in air at a concentration of 48 mg.m^{-3}, yielding a Ct-value of 240 mg.min.m^{-3} (0.3 LCt50 (96-h)). Mean values with s.e.m. (n=4, unless stated otherwise) are also presented.

Animal #	Time (min)	Concentration 7-SM-gua in tissue (number of adducts per 10^7 nucleotides)					
		Blood	Liver	Lung	Spleen	Bone marrow	Small intestine
HT58	10	4.1	n.d.	0.5	n.d.	n.d.	n.d.
HT59	10	2.7	n.d.	0.2	n.d.	n.d.	n.d.
HT60	10	0.9	n.d.	0.3	n.d.	n.d.	n.d.
HT61	10	6.4	n.d.	3.0	n.d.	n.d.	n.d.
Mean ±	s.e.m.	3.5 ± 1.2	n.d.	1.0 ± 0.7	n.d.	n.d.	n.d.
HT54	2880	5.4	n.d.	0.1	n.d.	n.d.	n.d.
HT55	2880	10.9	n.d.	n.d.	n.d.	n.d.	n.d.
HT56	2880	3.0	n.d.	n.d.	n.d.	n.d.	n.d.
HT57	2880	3.8	n.d.	n.d.	n.d.	n.d.	n.d.
		5.8 ± 1.8	n.d.	0.02 ± 0.02	n.d.	n.d.	n.d.

n.d. = not detectable

III.8.d. Distribution of the major adduct of sulfur mustard to DNA within the respiratory tract
Four anesthetized hairless guinea pigs were nose-only exposed to 1 LCt50 SM vapor in 5 min. After 4 h the respiratory tract, from the tip of the nose down to the lungs, was sampled. The respiratory tract was then divided into 6 regions, i.e., nasal organ, nasopharynx, larynx, trachea, carina and lung. DNA was extracted from these tissue samples, and the concentration of 7-SM-gua was measured with the immunoslotblot assay. The results are presented in Table 21 and Figure 26.

Table 21. Concentration (number of adducts/10^7 nucleotides) of 7-SM-gua in various parts of the respiratory tract of restrained, anesthetized hairless guinea pigs nose-only exposed to SM vapor in air at a concentration of 160 $mg.m^{-3}$ for 5 min, yielding a Ct-value of 800 $mg.min.m^{-3}$ (1 LCt50 (96-h)), at 4 h after ending the exposure. Mean values with s.e.m. (n=4, unless stated otherwise) are also presented.

Animal #	Time (h)	Concentration 7-SM-gua (number of adducts per 10^7 nucleotides)					
		Nasal organ	Naso-pharynx	Larynx	Trachea	Carina	Lung
GS1	4	30	55	89	91	§	0.4
GS2	4	57	§	85	93	57	0.09
GS3	4	86	90	92	86	64	0.6
GS4	4	49	61	88	90	36	0.3
Mean ±	s.e.m.	56 ± 12	69 ± 11[a]	88 ± 1	90 ± 2	52 ± 8[a]	0.3 ± 0.1

n.d. = not detectable
§ = DNA could not be isolated from the sample
[a] n=3

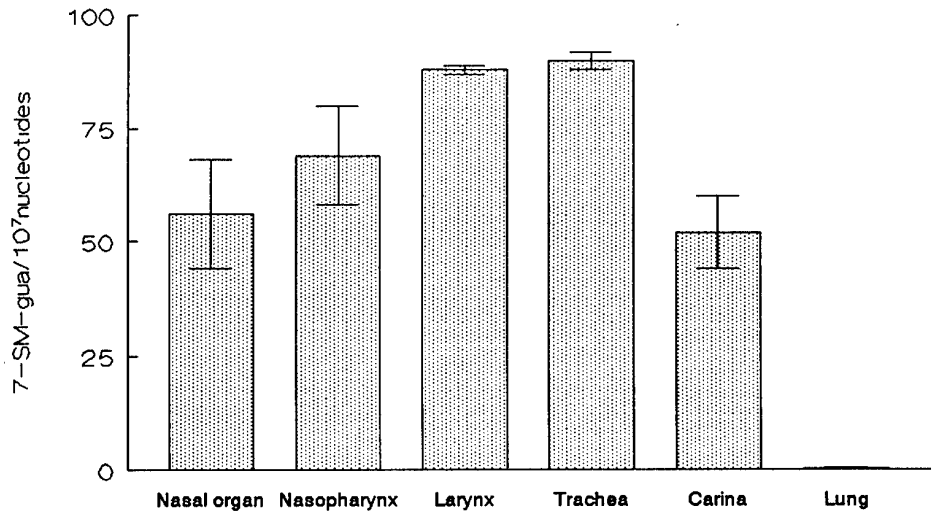

Figure 26. Mean 7-SM-gua concentrations (number of adducts per 10^7 nucleotides, ± s.d., n=4; n=3 for nasopharynx and carina) in various parts of the respiratory tract of 4 hairless guinea pigs 'nose-only' exposed to 1 LCt50 SM in 5 min, at 4 h after exposure. Nasal organ: epithelial cells of region of 'vomero nasal organ' and 'concha nasi ventralis'.

These data presented in Table 21 and Figure 26 clearly demonstrate that most adduct formation has occurred in the larynx and trachea, whereas almost no SM appears to have reached the lung.

III.8.e. Histopathological damage in the respiratory tract

Eight restrained, anesthetized guinea pigs (400-500 g) were nose-only exposed to SM vapor in air with a concentration of 160 $mg.m^{-3}$ for 5 min. This yields a Ct of 800 $mg.min.m^{-3}$, which corresponds with 1 LCt50 (96-h). Only four animals survived for 96 h. These animals were anesthesized, the trachea was cannulated and lungs were dissected free from the thoracic wall. Lung fixation was performed by intratracheal instillation of 10% formalin under light pressure (i.e., 30 cm water). All tissues were processed using standard histological methods, sectioned at 3 µm, and stained with haematoxylin and eosin. These preparations were subjected to histopathological evaluation. The results are presented in Figures 27B, D, F and H. Corresponding slides obtained from non-exposed animals are presented in Figures 27A, C, E and G.

Some of the animals had only damage in the trachea but hardly any injury in the lung lobes. In the nose squamous metaplasia* of the nose epithelium was found as well as a moderate purulent intraepithelium and subepithelial inflammation. There was also focal dysplasia and focal necrosis of the epithelium. A moderate inflammation was found in the nasopharynx. An inflammation with multifocal dysplasia of the epithelium and multifocal squamous metaplasia was found in the larynx (Figure 27B versus 27A).
The trachea below the larynx showed a moderate diffuse suppurative tracheitis with squamous epithelial metaplasia and multifocal dysplasia of the epithelium (Figure 27D versus 27C). The alveolar tissue injury was unequally distributed throughout the lung lobes, some of which had hardly any damage. Mostly there was slight dysplasia of bronchial epithelium; sometimes purulence was found in the bronchial lumen. In other parts of the lungs a moderate broncheitis (mainly granulocyte infiltration) was noticed with severe epithelial dysplasia and more purulence in the bronchial lumen (Figure 27F versus 27E). In some lung lobes there was only a moderate presence of so-called "foamy" macrophages. In other lobes infiltration of large numbers of macrophages was visible around terminal bronchioli and in some places a severe alveolar histiocytosis (infiltration of phagocytes) was visible with macrophages containing eosinofilic material (Figure 27H versus 27G). In some bronchioli severe tissue destruction was observed with so-called giant cells (extra large polymorphonuclear macrophages) and purulence in the broncheolous lumen.

Next page:

Figure 27. Light micrographs of lower parts of the respiratory tract (trachea below the larynx, bronchus, bronchiolus plus alveolus) of non-exposed (control) guinea pigs (left panel: A, C, E, G), and of animals 96 h after a 5-min nose-only exposure to 1 LCt50 of SM (right panel: B, D, F, H). Panel B: larynx showing a severe dysplasia of the multilayer epithelium. Panel D: squamous metaplasia and multifocal dysplasia of tracheal epithelium; subepithelial edema; note that the cilia band has disappeared. Panel F: characteristic is the luminal obstruction of the bronchus and its severe epithelial dysplasia. The obstruction contains granulocytes, shedded epithelial cells and purulence. Panel H: showing a final branch of a bronchiolus into which alveolar ducts open, filled up with inflammatory cells, destructed epithelium and purulence.

* Pathological terms according to Dorland's Illustrated Medical Dictionary (24th edition, WB Saunders Company, Philadelphia and London, 1965): Metaplasia: the change in the type of adult cells in a tissue to a form which is not normal for that tissue; Suppurative: producing pus; Squamous: scaly or platelike; Dysplasia: abnormality of development; Purulence: being purulent (containing pus); Histiocytosis: infiltration of phagocytes.

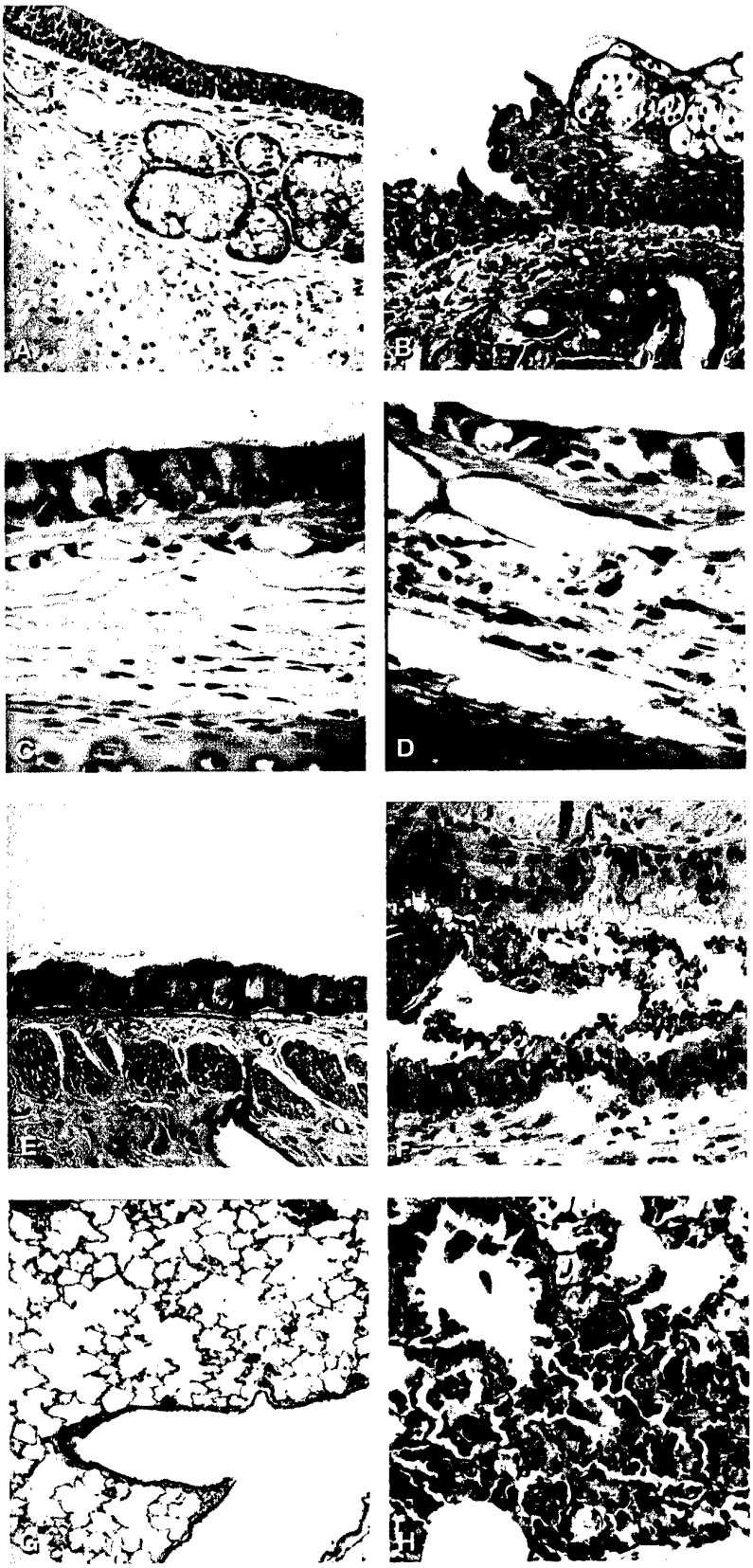

Figure 27. Legend: see previous page.

III.9 TOXICOKINETICS OF SULFUR MUSTARD AND ITS MAJOR DNA-ADDUCT IN ANESTHETIZED HAIRLESS GUINEA PIGS DURING AND AFTER 8-MIN NOSE-ONLY EXPOSURE TO 3 LCt50

III.9.a. Toxicokinetics of sulfur mustard in blood

Male hairless guinea pigs were anesthetized, a carotid canulla was inserted, and the animals were nose-only exposed to SM vapor in air at a concentration of 300 $mg.m^{-3}$ for 8 min. This yields a Ct-value of 2,400 $mg.min.m^{-3}$, i.e., 3 LCt50 for a 5-min exposure. Blood samples were taken just before the exposure, and at 0.5, 1, 2, 3, 5, 10, 15, 20, 30, 40, 60, 90, 120, 180 and 240 min after the start of the exposure. In order to avoid too much strain on each individual animal, sampling times were divided between two animals. As a result, 12 animals were used in order to obtain 6 values for each time point.

The mean SM vapor concentration to which the animals were exposed was 301 ± 4 $mg.m^{-3}$ (s.d., n=12). The mean Ct to which the animals were exposed in an 8-min time period was therefore 2,408 ± 32 $mg.min.m^{-3}$, which is well within the specified variation limits.

Blood samples were extracted with ethyl acetate to which the internal standard D_8-SM was added. The extracts were analyzed with the TDAS-GC-MS configuration (GLC configuration 4, *cf.* Section II.2). In nearly half of the samples SM could be measured. The mean concentrations of SM in blood, with standard error of the mean are presented in Table 22, whereas the concentrations as measured in the individual animals are presented in Table 23. The mean concentration-time course is shown in Figure 28.Obviously, the mean concentration-time course is rather capricious. So far, we have not succeeded in obtaining an adequate mathematic description for these data. The absorption phase can be reasonably well described with a mono-exponential equation, but a meaningful two-exponential fit of the distribution and elimination phases has not been obtained. The area under the curve (AUC), as calculated from 0 to 240 min with the trapezoidal approach, is 320 $ng.min.ml^{-1}$, which is 25-fold lower than the AUC calculated for 1 LD50 SM i.v..

Surprisingly,in half of the blood samples no SM could be detected, including all samples taken at 15 and 20 min, whereas in half of the samples taken at 60 min, SM was detected again. In two of the twelve animals SM could not be detected in any of the blood samples.

Table 22. Mean concentration in blood (ng/ml ± s.e.m., n=6 unless stated otherwise) of SM in anesthetized hairless guinea pigs at various time points during and after 8-min nose-only exposure to 300 $mg.m^{-3}$ SM, corresponding with 3 LCt50 (96-h; for a 5-min exposure).

Time	Concentration SM ± s.e.m. (ng/ml)
0	n.d
0.5	2.2 ± 0.7
1	1.2 ± 0.6
2	3.4 ± 0.4
3	3.0 ± 0.7
5	4.3 ± 1.6
10	0.9 ± 1.0 (n=5)
15	n.d.
20	n.d
30	0.6 ± 0.6
40	0.6 ± 0.7
60	0.9 ± 0.4
90	1.4 ± 0.8
120	1.2 ± 0.6
180	1.8 ± 0.6 (n=5)
240	1.7 ± 0.1 (n=5)

n.d. = not detectable (< 5 pg/ml)

Table 23. Concentrations in blood of SM (ng/ml) in individual anesthetized male hairless guinea pigs at various time points during and after 8-min nose-only exposure to SM vapor in air at a concentration of 300 $mg.m^{-3}$, yielding a Ct-value of 2,400 $mg.min.m^{-3}$, which corresponds with 3 LCt50 (96-h, for a 5-min exposure).

	Concentration of SM in blood (ng/ml) Hairless guinea pig #											
Time (min)	N1 (605)[a]	O1 (587)[a]	P1 (697)[a]	R1 (731)[a]	O2 (633)[a]	N2 (752)[a]	P2 (752)[a]	R2 (808)[a]	N3 (740)[a]	O3 (815)[a]	P3 (855)[a]	R3 (709)[a]
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0.5	3.94	*	3.75	*	*	n.d.	n.d.	*	2.90	*	2.63	*
1	*	3.05	*	1.91	n.d.	*	*	n.d.	*	n.d.	*	2.50
2	4.84	*	3.14	*	*	3.62	1.78	*	3.50	*	3.30	*
3	*	5.48	3.44	*	2.92	*	2.83	*	*	n.d.	3.61	*
5	*	*	*	7.50	*	*	*	n.d.	3.10	*	0.62	4.60
10	*	4.65	*	n.d.	n.d.	*	*	*	*	n.d.	*	n.d.
15	n.d.	*	n.d.	*	*	n.d.	n.d.	*	n.d.	*	n.d.	*
20	*	n.d.	n.d.	*	n.d.	*	n.d.	*	*	n.d.	n.d.	*
30	n.d.	*	*	3.47	*	n.d.	*	n.d.	n.d.	*	*	n.d.
40	*	n.d.	*	3.75	n.d.	*	*	n.d.	*	n.d.	*	n.d.
60	n.d.	*	2.18	*	*	n.d.	n.d.	*	1.20	*	2.02	*
90	*	1.35	*	4.90	2.15	*	*	n.d.	*	n.d.	*	n.d.
120	*	1.60	*	3.31	2.25	*	*	n.d.	*	n.d.	*	n.d.
180	1.29	*	1.72	*	*	3.74	n.d.	*	f.a.	*	2.38	*
240	1.40	*	2.03	*	*	1.81	1.41	*	f.a.	*	1.86	*

[a] Weight in grams

n.d. = Not detectable (< 5 pg/ml)

f.a. = Failed analysis

* = Not measured

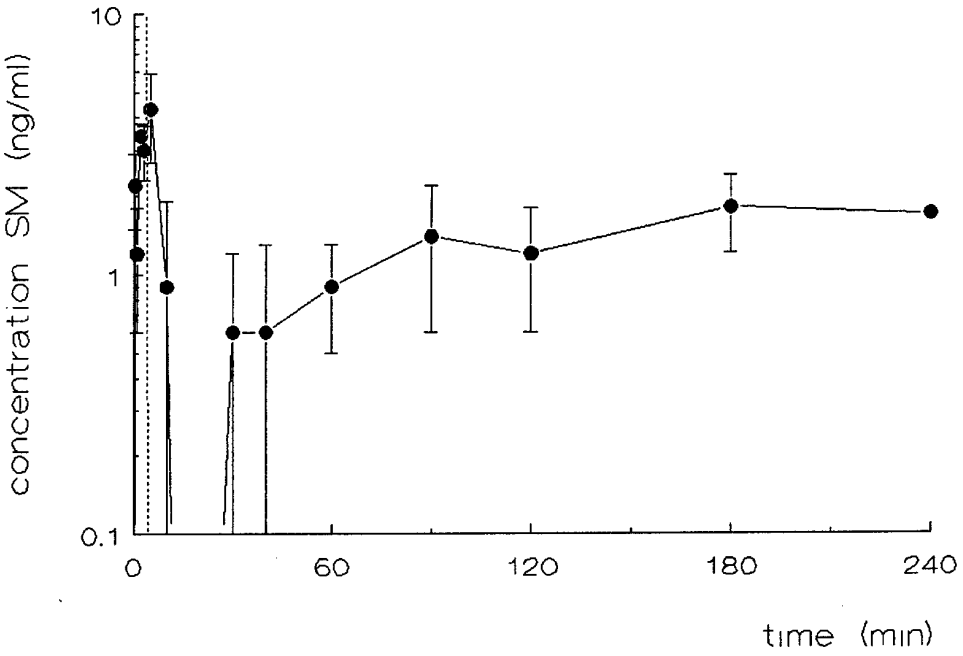

Figure 28. Mean concentration-time course of SM, with s.e.m. (n=5 or 6), in the blood of anesthetized hairless guinea pigs during and after 8-min nose-only exposure to 300 $mg.m^{-3}$, which corresponds with 3 LCt50 (96-h), for a 5-min exposure. The dotted line marks the end of the exposure period.

III.9.b. Distribution of intact sulfur mustard in tissues

Tissue concentrations of intact SM were measured in various tissues at three time-points after ending the nose-only exposure of hairless guinea pigs to a Ct of 2,400 $mg.min.m^{-3}$. The tissues were liver, lung, spleen, bone marrow and abdominal fat. The time-points were 10 min after ending the nose-only exposure, as well as halfway and at the end of the toxicokinetic experiment, i.e., 112 and 232 min after ending the exposure, respectively. The extracts of homogenized tissues in ethyl acetate were analyzed with GLC configuration 6 (*cf.* Section II.2). The results are presented in Table 24 as well as Figures 29 and 30.

Table 24. Concentration (ng/g) of intact SM in various tissues sampled at 10, 112 or 232 min after ending an 8-min nose-only exposure of anesthetized, restrained hairless guinea pigs to SM vapor in air at a concentration of 300 $mg.m^{-3}$, yielding a Ct of 2,400 $mg.min.m^{-3}$, which corresponds with 3 times the LCt50 (96-h) determined for 5-min exposure. Mean values with s.e.m. (n=4, unless stated otherwise) are also presented.

Animal #	Time (min)	Concentration SM in tissue (ng/g)				
		Liver	Lung	Spleen	Marrow	Fat
GvdS5	10	47.4	39.8	f.a.	71	f.a.
GvdS6	10	11.7	14.8	f.a.	7.2	f.a.
GvdS7	10	27.8	11.9	1.9	6.6	f.a.
GvdS8	10	24.9	1.3	n.d.	f.a.	f.a.
Mean ±	s.e.m.	28 ± 7	17 ± 8	1 ± 1[a]	28 ± 21[b]	
O1	112	0.82	n.d.	2.5	n.d.	f.a.
O2	112	f.a.	n.d.	n.d.	f.a.	f.a.
R1	112	8.9	n.d.	n.d	n.d.	f.a.
R3	112	f.a.	n.d.	n.d.	f.a.	f.a.
Mean ±	s.e.m.	5 ± 4[a]	n.d.	0.6 ± 0.6	n.d.[a]	
N1	232	f.a.	n.d.	6.6	n.d.	f.a.
N2	232	f.a.	f.a.	n.d.	n.d.	f.a.
P1	232	2.2	n.d.	n.d.	f.a.	f.a.
P3	232	4.7	f.a.	n.d.	n.d.	f.a.
N3	232	*	f.a.	*	*	*
Mean ±	s.e.m.	3.4 ± 1.2[a]	n.d.[a]	1.6 ± 1.6	n.d.[b]	

n.d. = not detectable (< 125 pg/g)
f.a. = failed analysis
* = not sampled
[a] n=2
[b] n=3

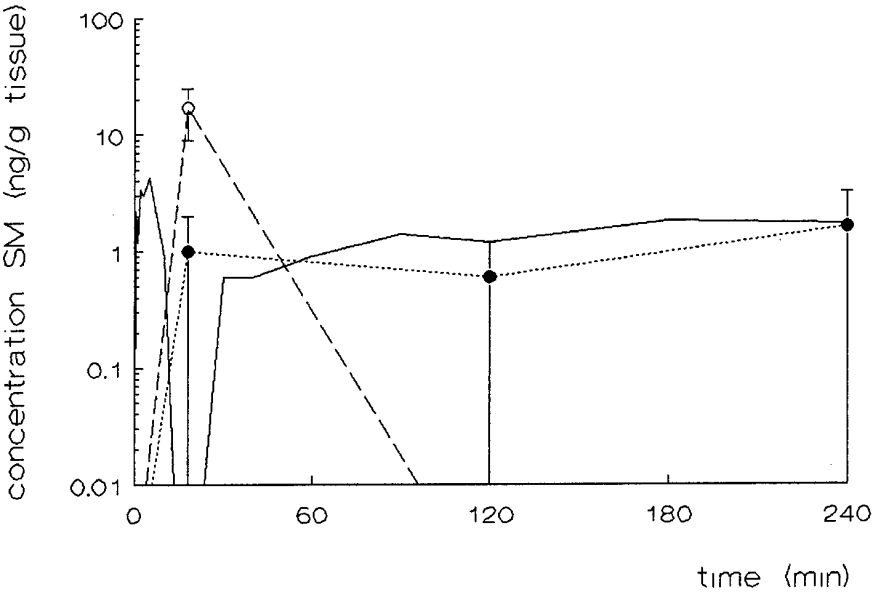

Figure 29. Concentration (ng/g, ± s.e.m.) of intact SM in lung (○) and spleen (●) of hairless guinea pigs at 10, 112 and 232 min after ending an 8-min nose-only exposure to SM vapor in air, yielding a Ct of 2,400 mg.min.m^{-3}, which corresponds with 3LCt50 (96-h) for a 5-min exposure. The solid line represents the concentration-time course of SM in blood (ng/ml).

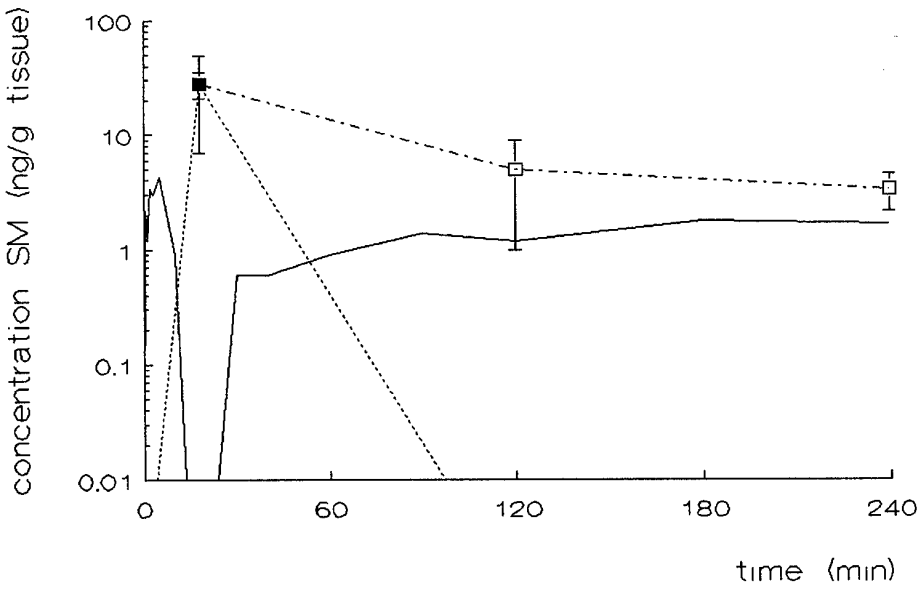

Figure 30. Concentration (ng/g, ± s.e.m.) of intact SM in liver (❑) and bone marrow (■) of hairless guinea pigs at 10, 112 and 232 min after ending an 8-min nose-only exposure to SM vapor in air yielding a Ct of 2,400 mg.min.m^{-3}, which corresponds with 3 LCt50 (96-h) for a 5-min exposure. The solid line represents the concentration-time course of SM in blood (ng/ml).

III.9.c. Toxicokinetics of the major adduct of sulfur mustard to DNA

Tissues were sampled from anesthetized and restrained male hairless guinea pigs at 10, 112 and 232 min after ending an 8-min nose-only exposure to a concentration of SM vapor in air of 300 $mg.m^{-3}$, yielding a Ct of 2,400 $mg.min.m^{-3}$. This exposure corresponds with 3 LCt50 (96-h) for a 5-min nose-only exposure. The tissues were blood, liver, lung, spleen, bone marrow and small intestine. DNA was isolated from tissues, after which the concentration of 7-SM-gua was determined with the immunoslotblot assay. The results are presented in Table 25 and Figures 31-33. Generally, only marginal adduct levels were found with large standard errors of the mean. The highest concentration of 7-SM-gua was found in the lung at 10 min after ending the exposure to 3 LCt50. At time points 112 and 232 min after ending the exposure, the concentration of 7-SM-gua was already decreasing.

Table 25. Concentration (number of adducts/10^7 nucleotides) of 7-SM-gua in various tissues at 10, 112, and 232 min after ending an 8-min nose-only exposure of restrained, anesthetized hairless guinea pigs to SM vapor in air at a concentration of 300 $mg.m^{-3}$, yielding a Ct-value of 2,400 $mg.min.m^{-3}$ (3 LCt50 (96-h) for a 5-min exposure). Mean values with s.e.m. (n=4, unless stated otherwise) are also presented.

Animal #	Time (min)	Concentration 7-SM-gua in tissue (number of adducts per 10^7 nucleotides)					
		Blood	Liver	Lung	Spleen	Bone marrow	Small intestine
GvdS5	10	*	n.d.	2.70	n.d.	n.d.	0.06
GvdS6	10	*	n.d.	0.75	n.d.	n.d.	n.d.
GvdS7	10	*	n.d.	0.31	n.d.	n.d.	n.d.
Gvds8	10	*	n.d.	0.04	n.d.	n.d.	n.d.
Mean ±	s.e.m.		n.d.	1.0 ± 0.6	n.d.	n.d.	0.02 ± 0.02
O1	112	0.07	n.d.	0.18	n.d.	0.03	n.d.
O2	112	§	0.33	1.05	0.02	n.d.	0.28
O3	112	0.14	n.d.	0.19	0.02	n.d.	n.d.
R1	112	0.15	n.d.	n.d.	0.01	n.d.	n.d.
R2	112	0.22	n.d.	0.03	0.01	n.d.	n.d.
R3	112	0.24	n.d.	0.55	n.d.	n.d.	n.d.
Mean ±	s.e.m.	0.16 ± 0.03[a]	0.06 ± 0.06[b]	0.3 ± 0.2[b]	0.01 ± 0.01[b]	0.005 ± 0.005[b]	0.05 ± 0.05[b]
P1	232	0.09	n.d.	n.d.	0.01	0.06	n.d.
P2	232	0.14	n.d.	n.d.	n.d.	n.d.	n.d.
P3	232	0.25	n.d.	0.18	0.05	n.d.	n.d.
N1	232	0.13	n.d.	n.d.	n.d.	0.01	n.d.
N2	2320	0.23	n.d.	0.18	0.05	n.d.	0.02
N3	2320	0.29	0.01	0.01	0.01	n.d.	n.d.
Mean ±	s.e.m.	0.19 ± 0.03[b]	0.002 ± 0.002[b]	0.06 ± 0.04[b]	0.02 ± 0.01[b]	0.01 ± 0.01[b]	0.003 ± 0.003[b]

* = Not sampled

n.d. = not detectable

§ DNA could not be isolated from the sample

[a] n=5

[b] n=6

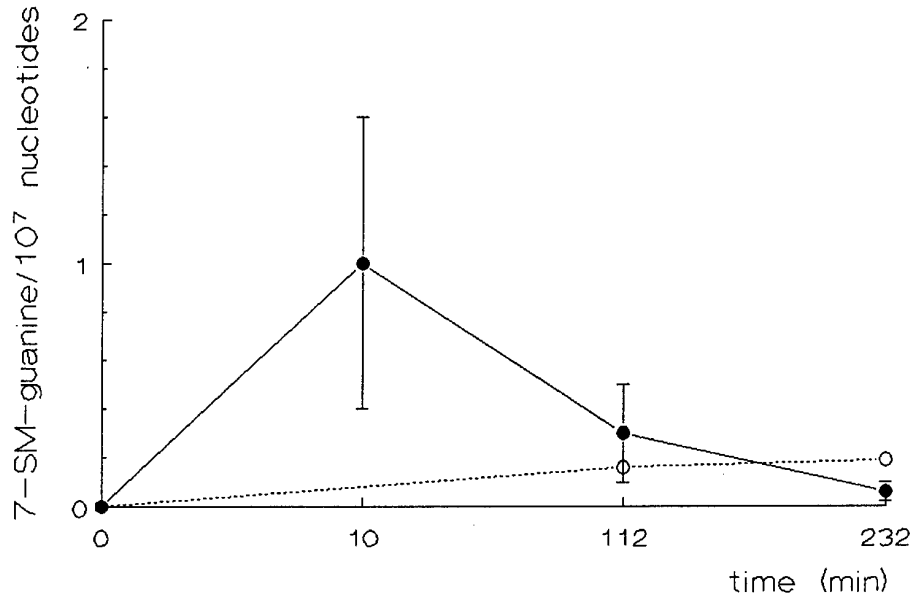

Figure 31. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± s.e.m., in blood (○) and lung (●) of hairless guinea pigs at various time points after ending an 8-min nose-only exposure to 3 LCt50 SM (96-h). The labels along the X-axis represent the time points (in min) after ending the exposure.

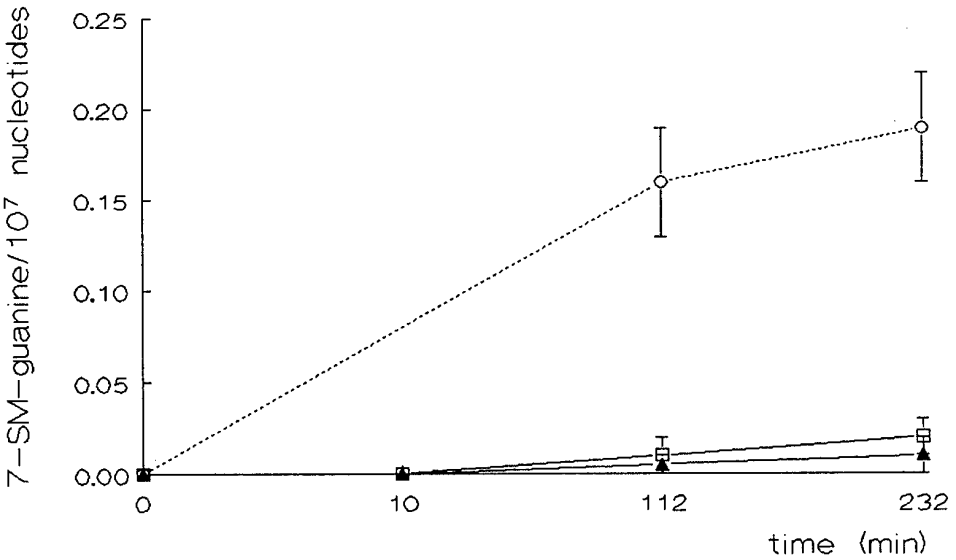

Figure 32. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± s.e.m., in blood (○) spleen (□) and bone marrow (▲) of hairless guinea pigs at various time points after ending an 8-min exposure to 3 LCt50 SM (96-h). The labels along the X-axis respresent the time points (in min) after ending the exposure.

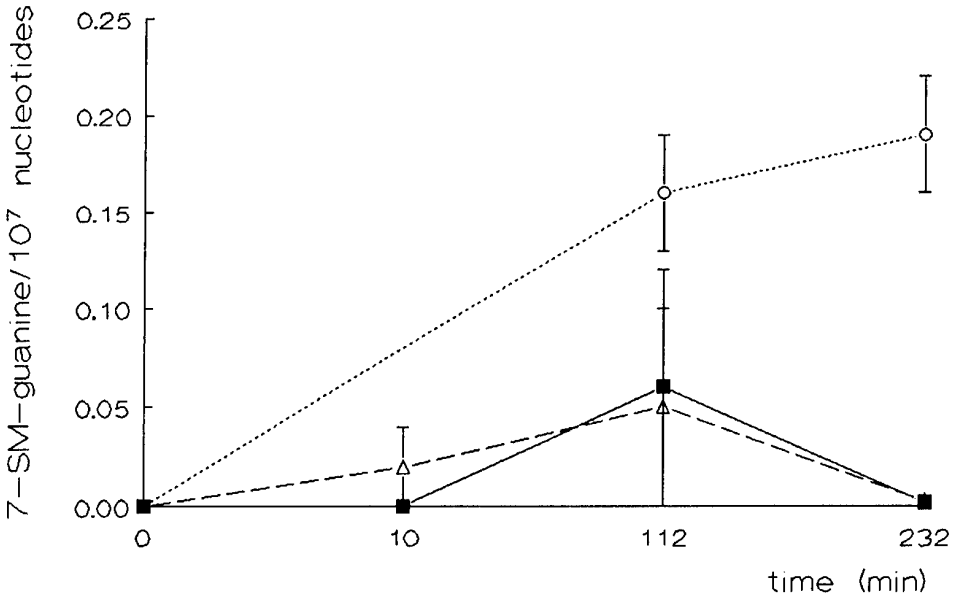

Figure 33. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± s.e.m., in blood (O), liver(■) and small intestine (Δ) of hairless guinea pigs at various time points after ending an 8-min nose-only exposure to 3 LCt50 SM (96-h). The labels along the X-axis respresent the time points (in min) after ending the exposure.

III.10 APPARATUS FOR PERCUTANEOUS EXPOSURE OF HAIRLESS GUINEA PIGS TO SULFUR MUSTARD VAPOR

An apparatus for whole-body exposure of hairless guinea pigs to SM vapor was designed. The apparatus should allow the animal to breathe clean air. A schematic representation of this design is shown in Figure 34.

The exposure unit consists of three major parts. The deepest part (on the right hand side of Figure 34) contains the inlet for the SM vapor in air (number 10 in Figure 34), which is connected to the vapor generation apparatus. The SM vapor in air is distributed through the exposure unit by means of a fan (number 5 in Figure 34). The main body of the exposure unit is connected to the deepest part via a metal ring with adjustable tension. An O-ring (number 14 in Figure 34) is fitted in between the two parts, ensuring an airtight connection. The main body contains the SM vapor exit (number 11 in Figure 34), a connection for a thermometer (number 13 in Figure 34) as well as a sampling point (number 12 in Figure 34), which is connected to the gas-sampling valve of a gas chromatograph, enabling frequent determination of the SM vapor concentration in the exposure unit.

The front panel (number 8 in Figure 34) of the exposure chamber is connected airtight to the main body via a metal ring with adjustable tension. An O-ring (number 14 in Figure 34) is fitted in between the two parts, ensuring an airtight connection. The metal grid on which the animal is fixated (number 2 in Figure 34) is mounted to the stainless steel front panel. The anesthetized animal is positioned with its nose in a rubber mask in the conical opening in the front panel through which the animal breathes clean air. With an adjustable fork (number 1 in Figure 34) positioned in the neck of the hairless guinea pig, the head of the animal is pushed firmly in the rubber mask to minimize the risk of leakage of the SM containing air to the exterior. In this prototype the carotid cannula (number 7 in Figure 34) protruded through a silicone rubber septum in the front panel.

The pressure in the exposure chamber is kept equal to that in the exterior or slightly higher. The exposure chamber is mounted on footrests and is placed in a perspex cabinet with air suction. The exposure chamber is constructed from glass and stainless steel. First, a polycarbonate prototype was made, to see whether the design was viable. Tests with smoke showed adequate mixing of air in the exposure chamber.

A procedure was developed for percutaneous exposure of hairless guinea pigs to SM vapor in air. The hairless guinea pig is anesthetized and fixated by taping its front and hind legs to the grid and by pushing its head into the rubber mask with the adjustable fork. The animal is then exposed to a stabilized concentration of SM vapor in vapor in air of *ca.* 285 mg/m^3 for *ca.* 35 min, yielding an exposure to 10,000 mg.min.m^{-3}, which is estimated to correspond with 1 percutaneous LCt50. At 5, 10 and 20 min during exposure blood samples are drawn via the carotid cannula. After 35 min the flow of SM vapor containing air is replaced with clean air. The animal remains in the exposure unit for the duration of the toxicokinetic experiment, which may be up to 4 h. Blood samples are drawn at eight or more post-exposure times. At the end of the experiment, the animal is euthanized with an overdose of anesthetic. The front panel, together with the grid on which the animal is positioned is placed in the front part of the cabinet. A chemical agent monitor (CAM) was used to ascertain that dangerous vapor concentrations were not generated from these parts of the exposure module and/or the animal. Subsequently, the ensemble was transported to a fume hood nearby, where the animal was dissected. The CAM was kept in the fume hood, next to the position where the experimentor was dissecting the animal.

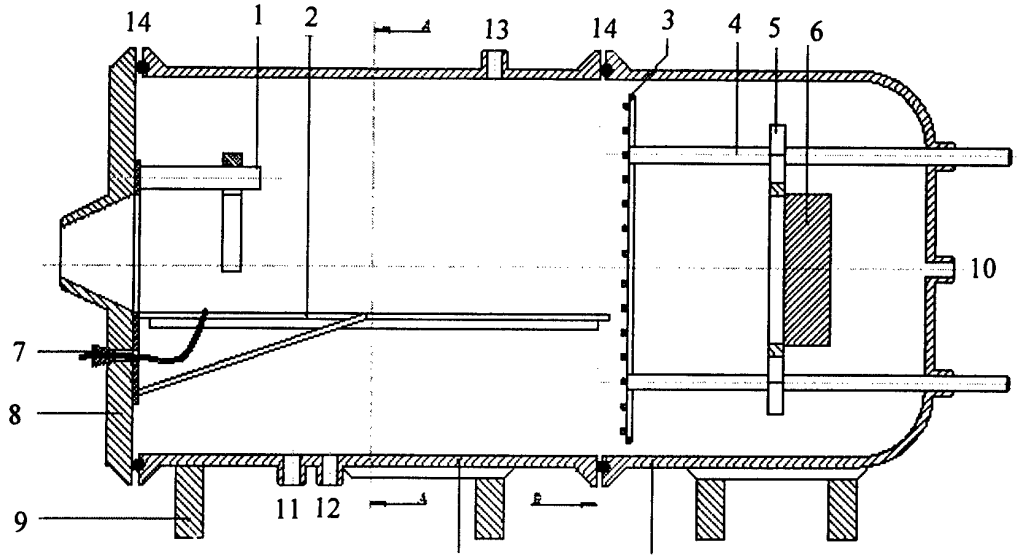

Figure 34. Schematic side-view of the prototype of the whole-body exposure chamber. (1) Fork for fixation of the anesthetized hairless guinea pig in the neck, (2) grid on which the animal is fixated, (3) grating of the fan, (4) mounting rail of the fan, (5) fan, (6) motor of the fan, (7) carotid cannula, (8) front panel of the exposure unit with opening through which the animal breathes clean air, (9) footrests of the exposure chamber, (10) inlet of SM vapor from generation apparatus, (11) SM vapor exit, (12) sampling point connected to GC, (13) input for thermometer, (14) O-rings.

The apparatus designed for whole-body exposure of hairless guinea pigs to SM vapor as well as the proposed procedures for the experiments were inspected by experts in the area of vapor generation from our laboratory. As suggested by them, additional overpressure valves were introduced into the vapor generation system.
After obtaining preliminary approval of the safety committee the apparatus as a whole was tested, first with water in the generation vial. Since rather high flow-rates (10 l/min) were needed in order to reach the required concentration of SM in the exposure chamber in a relatively short time period, some pressure build-up (*ca.* 40 mbar overpressure) was observed in the generation part of the configuration. Since the glass construction can bear at least 1 bar overpressure this was not considered as a reason for concern. The mercury-filled overpressure safety device, however, appeared to be insufficient for this overpressure. Therefore, it was replaced with an adjustable overpressure valve. The generation vial appeared to be too small to allow generation of the required high concentrations of SM at a high flow-rate. It was replaced by a larger size generation vial which can contain *ca.* 20 ml of SM. Furthermore, the capacity of the pump which sucks air from the exposure chamber through activated carbon filled canisters appeared to be inadequate. A pump with a higher capacity was purchased and installed.
The safety committee reviewed the modified apparatus as well as the proposed procedures again and written approval was obtained. Then, the generation vial was filled with neat SM and the apparatus was tested with live agent.

At the start of the exposure, the exposure chamber contains clean air. It takes several minutes to build up the required concentration of SM vapor inside the chamber. Likewise, it takes several minutes before the SM concentration in the chamber approaches zero after ending the exposure. The Ct-value of exposure is therefore determined as the area under the curve describing the time course of the SM concentration in the exposure chamber. The SM concentration is measured every 13 seconds by gas chromatography, via a gas sampling valve with a loop of 100 µl.

In a first pilot experiment a hairless guinea pig was anesthetized, a carotid artery cannula was inserted, and the animal was immobilized on the grid of the exposure chamber. Figure 35 shows a photograph of a hairless guinea pig inside the exposure chamber. Next, the exposure with SM vapor was started. The target SM vapor concentration was reached in *ca.* 8 min. From the actual SM concentrations at steady state the exposure duration needed for a Ct-value of 10,000 $mg.min.m^{-3}$ was calculated. At 35 min after starting the exposure, clean air was led into the exposure chamber, upon which the SM vapor concentration decreased to zero in *ca.* 8 min. From the concentration-time profile a Ct-value of 9,963 $mg.min.m^{-3}$ was calculated, which deviates only -0.4 % from the target value. Blood samples were taken at 30, 60, 90 and 120 min after the start of the exposure. An SM concentration of 84 ng/ml was measured in the blood sample taken at 30 min. In the samples that were drawn later the concentration was much higher, in view of the fact that the mass-spectrometer indicated 'overload'. It seemed highly unlikely that such high SM concentrations in the blood were actually the result of agent penetrating through the skin on such a small time scale. An artefact was therefore suspected. Figure 34 shows that the carotid cannula is exposed to SM vapor in the exposure chamber. Since the cannula is made of polyethylene it can be anticipated that SM will be absorbed by this material and then diffuses into the blood or saline in the cannula. Indeed, when a cannula filled with saline and closed at both ends was placed inside the chamber during the generation of SM vapor, very high concentrations of SM were recovered in the saline. We could not find material which is both inert towards SM and suitable as a cannula. As an alternative, we ensured that the cannula would not be exposed to SM vapor by leading the cannula from the carotid artery via the larynx and the nasal cavity to the clean environment outside the chamber. A photograph of a hairless guinea pig with the cannula protruding from the nose is shown in Figure 36.

In a second pilot experiment this approach was followed. Blood samples were taken at 30 and 60 min after starting the exposure. In these samples SM concentrations of 14.9 and 1.7 ng/ml were measured, respectively. The eyes of the animal were not covered in this experiment. Some absorption of SM via the eyes during exposure cannot be excluded for this experiment, which may explain the relatively high concentration in the blood sample taken at 30 min. At 90 min, the animal appeared to be dead.

In a third pilot experiment the eyes of the animal were covered with a small piece of US Marine Corps protective clothing. In blood samples taken at 5, 20 and 30 min after starting the exposure the SM concentration was below the detection limit (< 5 pg/ml). At 60 min, the animal appeared to be dead. A subsequent pilot experiment was performed in a similar way. In blood samples taken at 5 and 20 min after starting the exposure, the SM concentration was below the detection limit, at 30 min the concentration was 2.8 ng/ml. Unfortunately, the animal was dead at 60 min. We were puzzled by the death of the majority of the animals within 1 h. Since the same anesthesia was used throughout the various toxicokinetic studies of this Cooperative Agreement it seemed unlikely that the premature death of the animals was caused by the anesthesia. We wondered whether the cannula obstructed the airways in such a way that the animals suffocated. However, an anesthetized animal without a cannula, which was strapped onto the grid of the exposure chamber also died in *ca.* 40 min. We noticed that the ears of the immobilized animals turned pale as time progressed, which suggested that the fixation fork was positioned too tight over the neck of the animal, thus obstructing essential blood flows. The fork was adapted to solve this problem. However, the fork appeared not to be the cause for the untimely death of the animals. Finally, their death appeared to a related to the vigorous flushing of the exposure chamber with clean air after ending the exposure. Apparently this action caused excessive stress to the animals. Flushing the chamber with a gentle stream of humidified (> 70 %) clean air, under the same flow and pressure conditions as applied for SM vapor containing air, appeared to solve this problem.

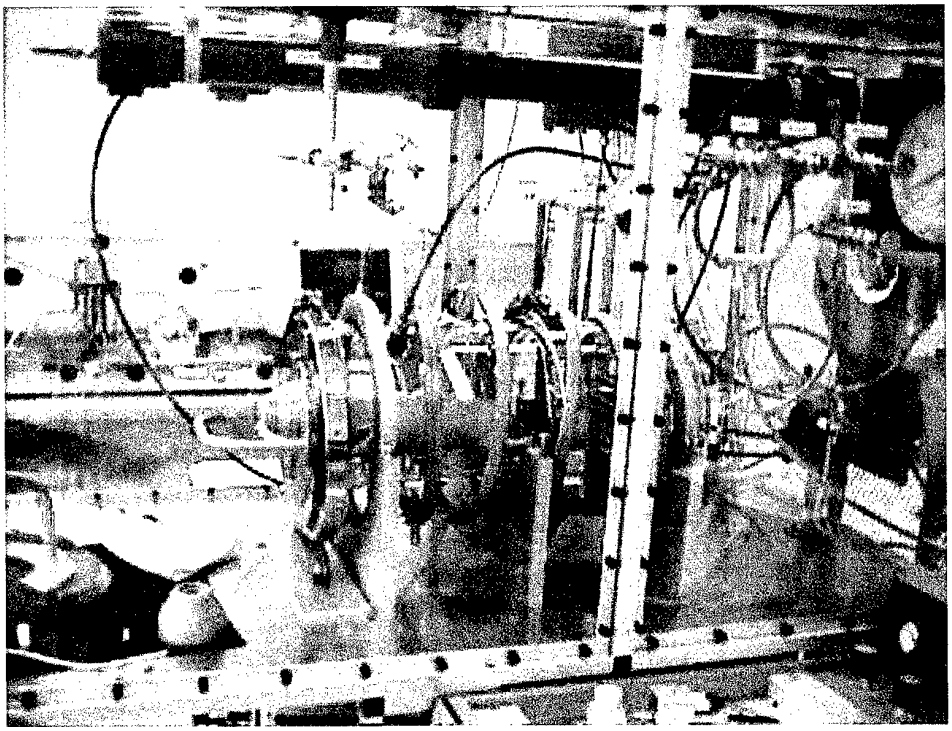

Figure 35. Photograph of a hairless guinea pig in the chamber for percutaneous exposure.

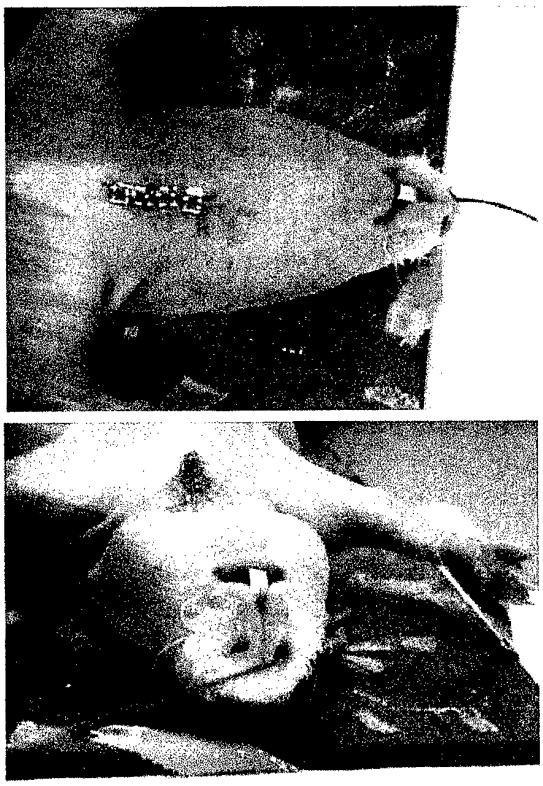

Figure 36. Photograph of a hairless guinea pig with a carotid artery cannula which is led via the larynx and the nasal cavity.

III.11 TOXICOKINETICS OF SULFUR MUSTARD IN ANESTHETIZED HAIRLESS GUINEA PIGS DURING AND AFTER PERCUTANEOUS EXPOSURE TO A Ct OF 10,000 mg.min.m^{-3}, WHICH CORRESPONDS WITH APPROXIMATELY 1 LCt50

III.11.a. Toxicokinetics of sulfur mustard in blood

Anesthetized and restrained hairless guinea pigs were whole-body exposed to an SM vapor concentration of 238 ± 4 mg.m^{-3} for 44.9 ± 0.7 min, resulting in an exposure of 10,700 ± 200 mg.min.m^{-3}. Blood samples were taken just before starting the exposure and at 5, 10, 20, 30, 40, 50, 60, 90, 120, 180, 210, 240 min after the start of the exposure. The volume of the blood samples ranged from 0.5 to 2 ml for the samples up to 210 min, and was 5 ml for the 240-min samples. The samples were extracted with ethyl acetate containing D_8-SM. The extracts were analyzed with gas chromatographic configuration 4 (TDAS-GC-MS, *cf.* Section II.2). Since the duration of the exposure was *ca.* 45 min, the time-points of 50 min up to 240 min were drawn after the exposure had ended.

In order to avoid too much strain on each individual animal, the sampling times were divided into two series. In theory, 12 animals would have to be used in order to obtain six values for each time-point. A total number of 15 animals had to be used, because occasionally a sample was lost due to analytical problems, or an animal died prematurely.

The mean concentrations in blood of SM with standard error of the mean are listed in Table 26, whereas the concentrations measured in the individual animals are presented in Table 27. A graphic representation of the mean concentration-time course is presented in Figure 37.

The datapoint obtained for hairless guinea pig S2 at time 30 min was suspected to be an outlyer. Application of the Q-test (Dean and Dixon, 1951) confirmed this suspicion. This datapoint was therefore rejected.

Table 26. Mean concentration in blood (ng/ml. ± s.e.m.; n=6, unless stated otherwise) of SM in anesthetized hairless guinea pigs at various time points during and after a *ca.* 45-min percutaneous exposure to SM vapor, yielding a Ct of 10,000 mg.min.m^{-3}, corresponding with approximately1 LCt50.

Time (min)	Concentration of SM (ng/ml blood, ± s.e.m.)	
0	n.d.	
5	n.d.	
10	1.0 ± 0.9	
20	1.6 ± 1.6	
30	3.3 ± 1.6	(n=5)
40	12 ± 5	
50	1.5 ± 0.6	
60	0.3 ± 0.1	
90	0.9 ± 0.7	
120	1.5 ± 0.8	
180	2.6 ± 0.6	
210	1.3 ± 0.6	(n=4)
240	0.9 ± 0.2	

n.d. = not detectable (< 5 pg/ml)

Table 27. Concentrations in blood (ng/ml) of sulfur mustard in individual anesthetized and restrained hairless guinea pigs at various time points during and after whole-body exposure to 10,000 mg.min.m^{-3} in *ca.* 45 min, corresponding with approximately 1 LCt50 (p.c.). Throughout the experiment the animals breathed clean air.

Time (min)	Concentration of sulfur mustard in blood (ng/ml) Hairless guinea pig #														
	U1 (809)[a]	W1 (934)[a]	S2 (767)[a]	T2 (727)[a]	U2 (736)[a]	W2 (701)[a]	S3 (833)[a]	T3 (882)[a]	W3 (885)[a]	S4 (904)[a]	T4 (869)[a]	U4 (921)[a]	W4 (999)[a]	T5 (777)[a]	U5 (754)[a]
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5	n.d.	n.d.	*	n.d.	n.d.	*	*	n.d.	f.a.	*	*	*	*	n.d.	*
10	*	*	5.7	*	*	n.d.	*	*	*	n.d.	n.d.	n.d.	0.50	*	*
20	n.d.	n.d.	*	n.d.	9.4	*	*	*	0.13	*	0.17	*	*	*	*
30	n.d.	8.7	82[b]	*	*	2.7	0.58	4.4	*	*	*	*	*	*	*
40	*	*	*	18.0	32	*	*	*	2.3	n.d.	*	f.a.	4.8	*	13.9
50	*	*	*	*	*	2.6	0.43	f.a.	*	n.d.	1.0	*	4.0	0.80	*
60	*	*	*	n.d.	0.80	*	*	*	f.a.	n.d.	0.08	0.30	*	0.66	*
90	*	*	*	4.4	1.2	*	*	*	n.d.	n.d.	0.002	*	*	*	n.d.
120	*	*	*	5.1	1.4	*	*	*	0.07	n.d.	0.05	*	2.50	*	*
180	*	*	*	*	*	3.0	5.6	1.7	*	*	*	f.a.	2.03	1.06	1.9
210	*	*	*	*	*	1.5	f.a.	0.18	*	*	*	f.a.	2.84	0.83	f.a.
240	*	*	*	*	*	1.4	1.4	0.05	*	*	*	f.a.	0.82	0.67	1.3

[a] Weight in grams
[b] Datapoint rejected
n.d. = Not detectable
f.a. = Failed analysis
* = Not measured

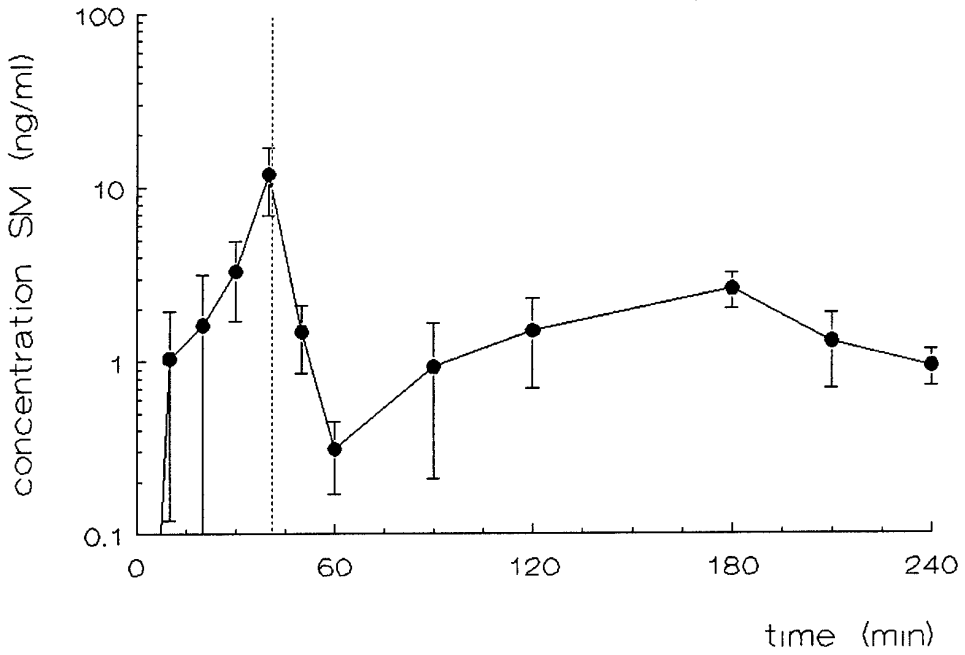

Figure 37. Mean concentration-time course (ng/ml, ± s.e.m.) of SM in blood of anesthetized, restrained hairless guinea pigs during and after percutaneous exposure to a Ct of 10,000 mg.min.m^{-3} in *ca.* 45 min, corresponding with approximately 1 LCt50 (p.c.). The dotted line marks the end of the exposure period.

From Table 26 and Figure 37 it is clear that during the exposure the concentration of SM in blood increases gradually. After ending the exposure, the concentration first decreases, reaches a minimum at time-point 60 min and increases again up to time-point 180 min. Subsequently, the concentration decreases again. This pattern shows a considerable similarity to that observed after 8-min nose-only exposure of hairless guinea pigs to a Ct of 2,400 mg.min.m^{-3} (*cf.* Section III.9.a).

III.11.b. Distribution of intact sulfur mustard in tissues

Tissue concentrations of intact SM were measured in liver, lung, spleen, bone marrow, abdominal fat, and dorsal skin at three time-points after ending the percutaneous exposure. The time-points were 10 min after ending the whole-body exposure, halfway, and at the end of the toxicokinetic experiment, i.e., at 120 and 240 min after starting the exposure, coresponding with 75 and 195 min after ending the exposure, respectively. The tissues were homogenized by means of an ultra-thurrax in ethyl acetate to which the internal standard D_8-SM was added. The ethyl acetate extracts were analyzed with GLC configuration 6 (TCT-GC-PFPD, *cf.* Section II.2). The results are presented in Table 28 and in Figures 38-40.

Already at 10 min after ending the exposure considerable concentrations of SM were measured in all tissues studied. By far the highest concentrations were observed in skin, which is not surprising in view of the high external load on the body of the animal.

Table 28. Concentration (ng/g) of intact SM in various tissues sampled at 10, 75 or 195 min after ending a 45-min whole-body exposure of anesthetized, restrained hairless guinea pigs to SM vapor, yielding a Ct of 10,000 mg.min.m^{-3}, which corresponds with approximately 1 percutaneous LCt50. Mean values with s.e.m. (n=4, unless stated otherwise) are also presented.

Animal #	Time (min)	Concentration of SM in tissue (ng/g)					
		Liver	Lung	Spleen	Marrow	Fat	Skin
SMper1	10	20.5	n.d.	2.4	8.6	f.a.	57.9
SMper2	10	50.5	19.6	*	217	2.6	34.3
SMper3	10	243	22.4	75.7	32.6	3.3	1387
SMper4	10	f.a.	f.a.	f.a.	186	3.7	f.a.
Mean ±	s.e.m.	105 ± 70[a]	14 ± 7[a]	39 ± 37[b]	111 ± 53	3.2 ± 0.3[a]	490 ± 390[a]
T2	75	f.a.	f.a.	f.a.	f.a.	n.d.	f.a.
U2	75	f.a.	f.a.	f.a.	f.a.	n.d.	f.a.
T4	75	f.a.	f.a.	f.a.	f.a.	f.a.	f.a.
W3	75	f.a.	f.a.	f.a.	f.a.	f.a.	f.a.
Mean ±	s.e.m.					n.d.[b]	
S3	195	f.a.	f.a.	f.a.	f.a.	1.4	26.3
T3	195	5.9	f.a.	n.d.	f.a.	n.d.	18.2
U4	195	f.a.	f.a.	f.a.	f.a.	f.a.	f.a.
W2	195	f.a.	f.a.	f.a.	f.a.	f.a.	f.a.
Mean ±	s.e.m.	5.9[c]		n.d.[c]		0.7 ± 0.7[b]	22 ± 4[b]

n.d. = not detectable (< 125 pg/g)
f.a. = failed analysis
* = no sample available
[a] n=3
[b] n=2
[c] n=1

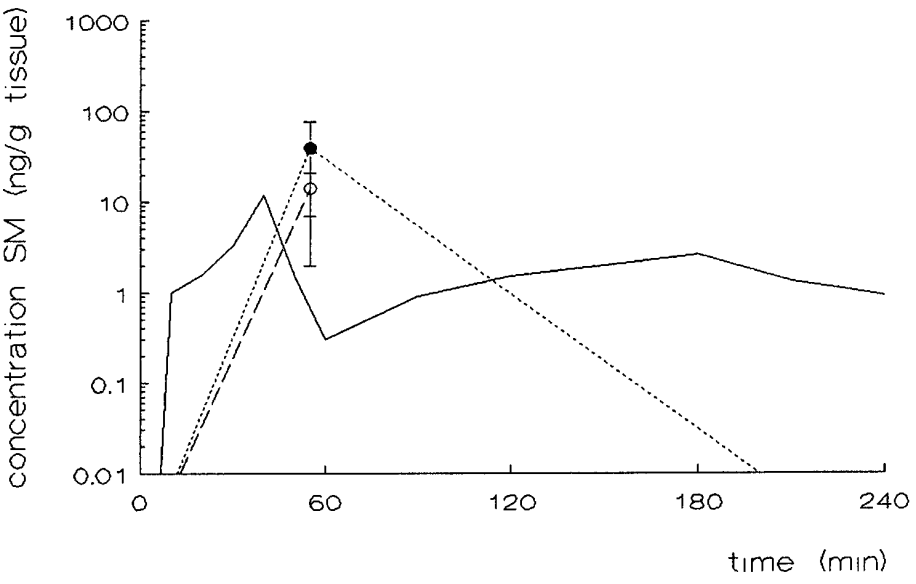

Figure 38. Concentration (ng/g, ± s.e.m.) of intact SM in lung (○) and spleen (●) of hairless guinea pigs at 10, 75 and 195 min after ending a 45-min percutaneous exposure to SM vapor in air, yielding a Ct of 10,000 mg.min.m^{-3}, which corresponds with approximately 1 LCt50. The solid line represents the concentration-time course of SM in blood (ng/ml).

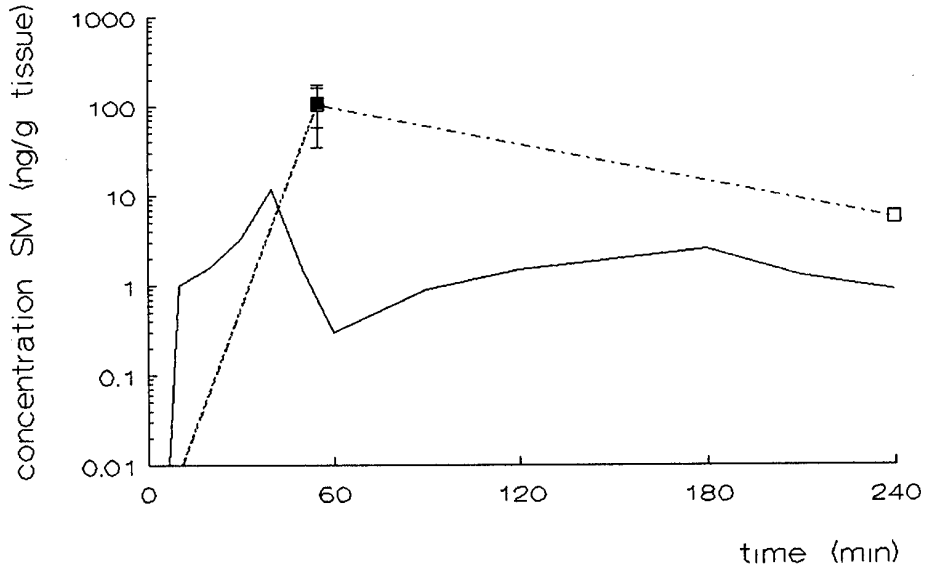

Figure 39. Concentration (ng/g, ± s.e.m.) of intact SM in liver (❑) and bone marrow (■) of hairless guinea pigs at 10, 75 and 195 min after ending a 45-min percutaneous exposure to SM vapor in air yielding a Ct of 10,000 mg.min.m^{-3}, which corresponds with approximately 1 LCt50. The solid line represents the concentration-time course of SM in blood (ng/ml).

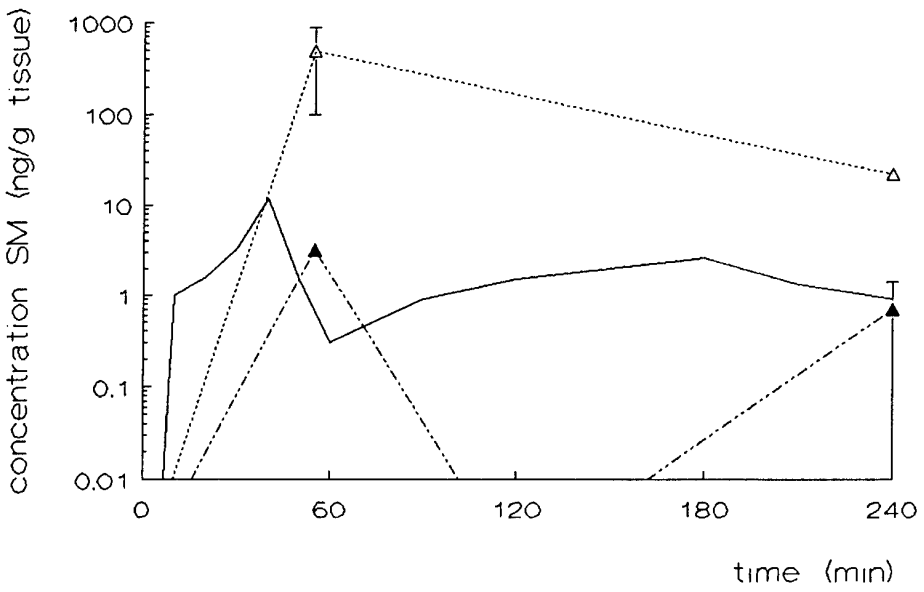

Figure 40. Concentration (ng/g, ± s.e.m.) of intact SM in fat tissue (▲) and skin (Δ) of hairless guinea pigs at 10, 75 and 195 min after ending a 45-min percutaneous exposure to SM vapor in air yielding a Ct of 10,000 mg.min.m^{-3}, which corresponds with approximately 1 LCt50. The solid line represents the concentration-time course of SM in blood (ng/ml).

III.11.c. Toxicokinetics of the major adduct of sulfur mustard to DNA

In conjunction with the toxicokinetic experiments described above, tissue samples were taken from five animals at 75 and 195 min after ending the 45-min exposure, which coincided with halfway and the end of the toxicokinetic experiments,respectively. Additional experiments were performed in order to sample tissues from 4 animals at 10 min and 24 h after ending the percutaneous exposure. Unfortunately, one animal did not survive for 24 h.

The tissues studied were blood, liver, lung, spleen, bone marrow, small intestine and (dorsal) skin. DNA was isolated from portions of these samples in order to perform the immunoslotblot assay. The results are presented in Table 29, and Figures 41-42.

The yield and purity of the DNA isolated from liver, lung, spleen, bone marrow, and small intestine were as usual. However, the yields of DNA from the skin samples were extremely low indicating very high adduct levels. Furthermore, the yield of DNA from the blood samples was very low in spite of the addition of the red blood cell lysis buffer immediately after collection of the blood.

Table 29. Concentration (number of adducts/10^7 nucleotides) of 7-SM-gua in various tissues at 10, 75, 195 and 1395 min after ending a 45-min whole-body exposure of anesthetized, restrained hairless guinea pigs to SM vapor, yielding a Ct of 10,000 mg.min.m^{-3}, which corresponds with approximately 1 percutaneous LCt50. Mean values with s.e.m. (n=4, unless stated otherwise) are also presented.

Animal #	Time (min)	Concentration of 7-SM-gua in tissue (number of adducts per 10^7 nucleotides)						
		Blood	Liver	Lung	Spleen	Bone marrow	Small intestine	Skin
SMper1	10	0.9	n.d.	0.29	0.12	0.06	0.02	§
SMper2	10	0.8	n.d.	0.02	0.04	0.02	0.03	§
SMper3	10	§	0.02	0.31	0.10	0.01	0.02	§
SMper4	10	§	0.01	0.38	0.08	n.d.	0.01	§
Mean ±	s.e.m.	0.85 ± 0.05[a]	0.01 ± 0.01	0.2 ± 0.1	0.08 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	§
S1	75	§	0.05	0.73	0.75	0.03	0.14	§
T2	75	0.6	0.04	0.84	0.46	0.06	0.01	§
U2	75	§	0.08	1.65	0.91	0.05	0.15	§
S4	75	§	0.10	n.d.	0.06	0.10	0.06	78
W3	75	§	0.01	n.d.	0.02	0.56	0.06	§
Mean ±	s.e.m.	0.6[b]	0.06 ± 0.02[c]	0.6 ± 0.3[c]	0.4 ± 0.2[c]	0.3 ± 0.2[c]	0.08 ± 0.03[c]	78[b]
W2	195	0.02	0.07	1.1	0.56	0.04	0.05	§
T3	195	n.d.	0.04	0.60	0.25	0.15	0.15	§
S3	195	0.16	n.d.	0.18	0.68	0.18	n.d.	§
U5	195	1.5	n.d.	n.d.	0.03	n.d.	n.d.	77
T5	195	1.3	n.d.	0.03	0.02	n.d.	n.d.	58
Mean ±	s.e.m.	0.6 ± 0.3	0.02 ± 0.01	0.4 ± 0.2	0.3 ± 0.1	0.07 ± 0.04	0.04 ± 0.03	68 ± 9[a]
SMper5	1395	n.d.	0.01	0.39	0.07	0.01	0.01	§
SMper7	1395	n.d.	n.d.	0.16	n.d.	0.03	0.01	§
SMper8	1395	n.d.	n.d.	0.06	0.02	0.03	n.d.	§
Mean ±	s.e.m.	n.d.[d]	0.003 ± 0.003[d]	0.2 ± 0.1[d]	0.03 ± 0.02[d]	0.02 ± 0.01[d]	0.007 ± 0.003[d]	§

n.d. = not detectable

§ = DNA could not be isolated from the sample

[a] n=2

[b] n=1

[c] n=5

[d] n=3

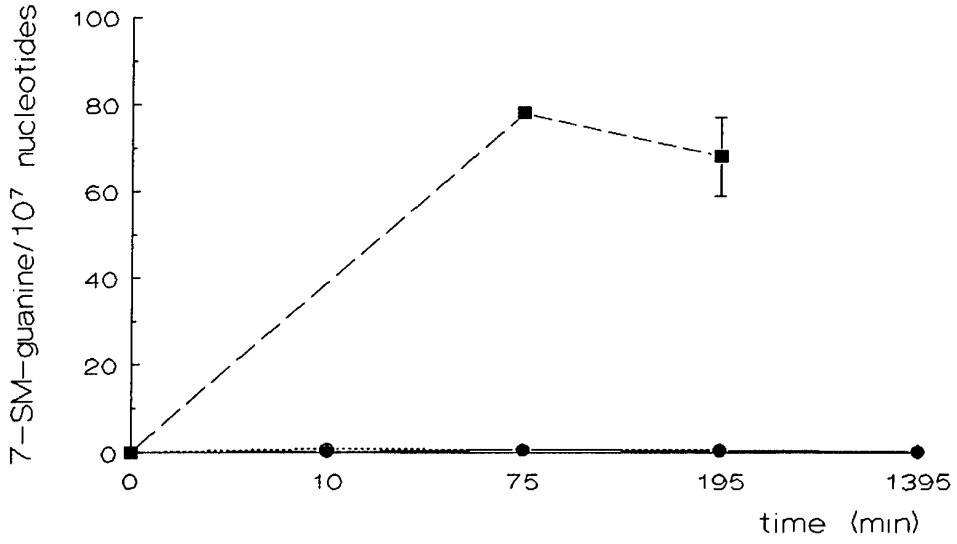

Figure 41. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± s.e.m., in blood (○), lung (●) and skin (■) of hairless guinea pigs at various time points after ending a 45-min percutaneous exposure to SM vapor, yielding a Ct of 10,000, which corresponds with approximately 1 LCt50. The labels along the X-axis represent the time points (in min) after ending the exposure.

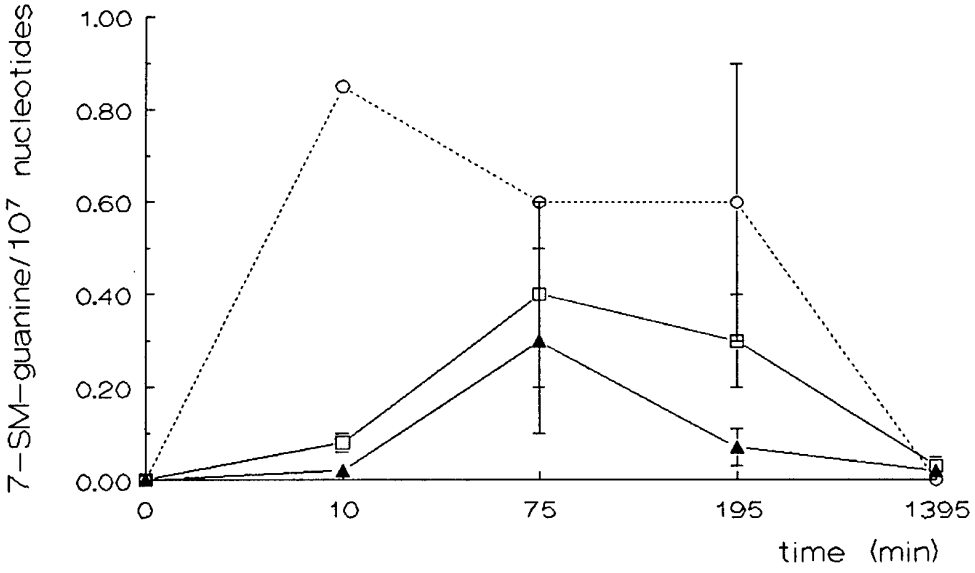

Figure 42. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± s.e.m., in blood (○), spleen (□) and bone marrow (▲) of hairless guinea pigs at various time points after ending a 45-min percutaneous exposure to SM vapor, yielding a Ct of 10,000, which corresponds with approximately 1 LCt50. The labels along the X-axis represent the time points (in min) after ending the exposure.

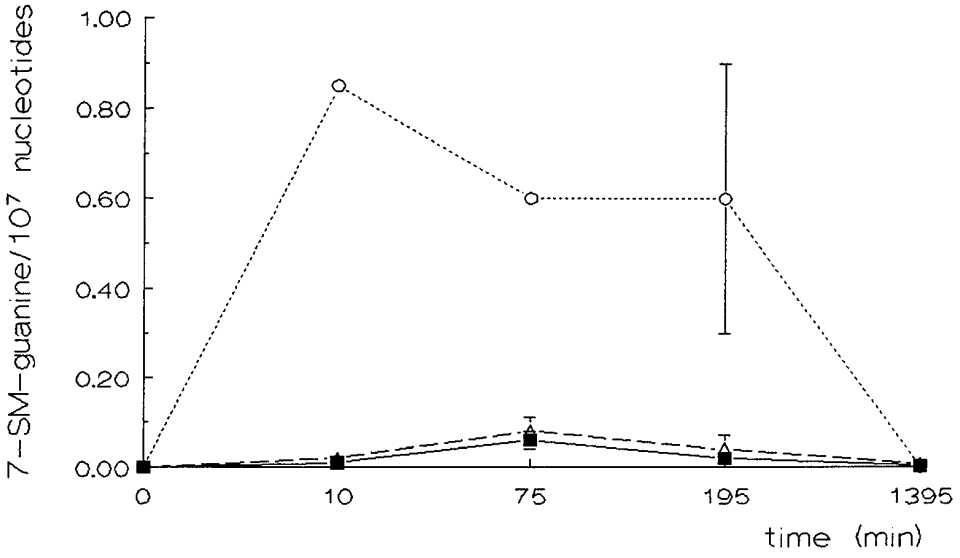

Figure 43. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± s.e.m., in blood (○), liver (■) and small intestine (Δ) of hairless guinea pigs at various time points after ending a 45-min percutaneous exposure to SM vapor, yielding a Ct of 10,000, which corresponds with approximately 1 LCt50. The labels along the X-axis represent the time points (in min) after ending the exposure.

III.12 PROTECTION OF HAIRLESS GUINEA PIGS AGAINST RESPIRATORY TOXICITY OF SULFUR MUSTARD BY SCAVENGER PRETREATMENT

III.12.a. Pretreatment with N-acetyl cysteine

The protective ratio of pretreatment with N-acetyl cysteine against respiratory exposure to SM was determined. Groups of six male hairless guinea pigs were anesthetized with ketamine. A solution of N-acetyl cysteine (NAC) in saline was administered i.p. at a dose of 5 mmoles/kg. Next, they were restrained in the modified Battelle tubes of the respiratory exposure apparatus. One min after administration of the scavengers, the animals were nose-only exposed to SM vapor in air for 5 min. Next, the animals were transferred to cages and were observed for 96 h, after which the 96-h mortality rate was determined. The obtained results are presented in Table 30.

Table 30. Number and percentage of dead animals at 96 h after nose-only exposure to various concentrations of SM vapor in air for 5 min. Animals were pretreated with N-acetyl cysteine (NAC, 5 mmol/kg, i.p.), 1 min prior to the challenge.

Ct-value of SM challenge ($mg.min.m^{-3}$)	LCt50 equivalent	pH NAC solution	Number of dead animals at 96 h/total number	Mortality (%)
813	1	1.7	5/6	83.3
1054	1.3	1.6	5/6	83.3
1610	2	1.6	6/6	100

According to the results shown in Table 30 pretreatment with NAC appears to increase the toxicity of SM. A challenge which corresponds with 1 LCt50 in untreated animals causes 83.3 % mortality in NAC-pretreated animals. This result seems very unlikely. However, a complication of an unexpected nature may have occurred. The technicians observed that the animals showed signs of discomfort upon i.p. administration of NAC. The pH of the NAC solution in saline appeared to be as low as 1.6. Therefore, irritation on the peritoneum is not surprising. It may well be that the absorption of the compounds was delayed by the low pH of the solutions.

Consequently, it seemed necessary to administer NAC in a solution with a pH closer to the physiological pH. A commercial preparation of NAC for parenteral administration is available, i.e., Fluimucil®. This preparation has a pH of 6.5 due to the addition of sodium hydroxide and disodium edetate.

We first repeated the challenge corresponding with 1.3 LCt50 SM after pretreatment of ketamine-anesthetized hairless guinea pigs with NAC in its pharmaceutical preparation Fluimucil®. For the Fluimucil®-pretreated group an improvement in protective efficacy was observed in comparison with the group pretreated with the acidic NAC solution, i.e., 3 out of 6 animals survived the challenge with 1.3 LCt50 instead of 1 out of 6. In view of this difference, a full challenge experiment was performed with Fluimucil®-pretreated animals. The results are presented in Table 31.

The LC50 of SM for 5-min nose-only exposure in NAC-pretreated hairless guinea pigs was calculated from the mortality data shown in Table 31 by probit analysis according to Litchfield and Wilcoxon (1949). The results of this probit analysis are presented in Table 32. The LC50 appeared to be 257 $mg.m^{-3}$, with a 95-% confidence interval of 154-422 $mg.m^{-3}$. This corresponds with an LCt50-value of 1285 $mg.min.m^{-3}$ (95-% confidence interval 770-2110 $mg.min.m^{-3}$). According to the Student's t-test, these LC50 and LCt50 values are not significantly different from those in non-pretreated animals, which are 160 $mg.m^{-3}$ (95-% confidence interval 140-184 $mg.m^{-3}$) and 800 $mg.min.m^{-3}$ (95-% confidence interval 700-920 $mg.min.m^{-3}$), respectively.

Table 31. Number and percentage of dead animals at 96 h after nose-only exposure to various concentrations of SM vapor in air for 5 min. Animals were pretreated with an i.p. dose of Fluimucil® corresponding with a N-acetyl cysteine dose of 5 mmol/kg, 1 min prior to the challenge.

Ct-value of SM challenge ($mg.min.m^{-3}$)	LCt50 equivalent	pH Fluimucil® solution	Number of dead animals at 96 h/total number	Mortality (%)
818	1	6.8	1/5	20
1034	1.3	6.8	3/6	50
1354	1.7	7.0	2/6	33.3
1615	2	7.0	4/6	66.7
1992	2.5	7.0	5/6	83.3

Table 32. LC10, LC30, LC50, LC70 and LC90 (96-h) with 95 % confidence limits, calculated via probit analysis, for 5-min nose-only exposure of anesthetized male hairless guinea pigs to sulfur mustard vapor in air. Animals were pretreated with Fluimucil® (N-acetyl cysteine, 5 mmol/kg, i.p.), 1 min prior to the challenge.

LC	$mg.m^{-3}$	95 % confidence limits ($mg.m^{-3}$)
10	128	2 - 187
30	193	30 - 251
50	257	154 - 422
70	343	264 - 2100
90	519	519 – 34583

III.12.b. Pretreatment with cysteine isopropyl ester

After studying the literature on the efficacy of cysteine esters (Lailey *et al.*, 1991) and deliberations with colleagues from the DERA, Porton Down, UK, it was decided to test the cysteine isopropyl ester as a scavenger for SM.

The protective ratio of pretreatment with CIPE against respiratory exposure to SM was determined. Groups of six male hairless guinea pigs were anesthetized with ketamine. A solution of CIPE in saline was administered i.p. at a dose of 5 mmoles/kg. Next, they were restrained in the modified Battelle tubes of the respiratory exposure apparatus. One min after administration of the scavengers, the animals were nose-only exposed to SM vapor in air for 5 min. Next, the animals were transferred to cages and were observed for 96 h, after which the 96-h mortality rate was determined. The obtained results are presented in Table 33.

Table 33. Number and percentage of dead animals at 96 h after nose-only exposure to various concentrations of SM vapor in air for 5 min. Animals were pretreated with cysteine isopropyl ester (CIPE, 5 mmol/kg, i.p.), 1 min prior to the challenge.

Ct-value ($mg.min.m^{-3}$)	LCt50 equivalent	pH CIPE solution	Number of dead animals at 96 h/total number	Mortality (%)
819	1	2.4	1/6	16.7
1028	1.3	2.5	1/6	16.7
1616	2	2.3	6/6	100

In view of the observed the pH-dependancy of the result of pretreatment with NAC, we wondered whether this would also be the case for CIPE. Therefore, we repeated the challenge experiments with a CIPE solution of which the pH was raised to *ca.* 7 with disodium edetate and sodium hydroxide. The results of these experiments are presented in Table 34.

Table 34. Number and percentage of dead animals at 96 h after nose-only exposure to various concentrations of SM vapor in air for 5 min. Animals were pretreated with cysteine isopropyl ester (CIPE, 5 mmol/kg, i.p.), 1 min prior to the challenge.

Ct-value ($mg.min.m^{-3}$)	LCt50 equivalent	pH CIPE solution	Number of dead animals at 96 h/total number	Mortality (%)
807	1	6.9	4/6	66.7
1054	1.3	6.2	2/6	33.3
1176	1.45	6.9	4/6	66.7
1340	1.65	7.0	4/6	66.7
1591	2	7.0	6/6	100

Probit analysis was applied to the mortality rates shown in Table 34 in order to obtain LC50 and LCt50 values for 5-min nose-only exposure to SM in CIPE-pretreated animals. Unfortunately, a significant regression could not be obtained, which is mainly due to the high mortality rate at 800 $mg.min.m^{-3}$, and also due to the fact that three doses lead to the same mortality rate. In the probit analysis according to Litchfield and Wilcoxon (1949) a 100 % mortality rate is substituted with a slightly lower value, since a 100-% response cannot be transformed into a probit. The substitute value is found by performing linear regression on the probit values of the logarithms of the doses which produce responses between 0 and 100 %. Next, the probit value which corresponds with the log dose of the 100-% response datapoint is determined with the linear equation, and is subsequently replaced by a corrected value. In the case of CIPE only 4 datapoints can be used, of which 3 have the same response. The linear equation fitted to these datapoints had a correlation coefficient as low as 0.04, which is not a significant regression. In order to be able to conclude whether CIPE significantly protects hairless guinea pigs against a respiratory challenge with SM it was decided to perform a probit analysis on 4 out the 5 obtained data, i.e., by omitting the result observed for exposure to 800 $mg.min.m^{-3}$. The results are presented in Table 35.

Table 35. LC10, LC30, LC50, LC70 and LC90 (96-h) with 95 % confidence limits, calculated via probit analysis, for 5-min nose-only exposure of anesthetized male hairless guinea pigs to sulfur mustard vapor in air. Animals were pretreated with cysteine isopropyl ester (CIPE, 5 mmol/kg, i.p.), 1 min prior to the challenge.

LC	$Mg.m^{-3}$	95 % confidence limits ($mg.m^{-3}$)
10	175	20- 207
30	203	63- 228
50	225	133- 255
70	250	215- 376
90	289	255-1109

The calculated LC50 for SM after pretreatment with CIPE is 225 $mg.m^{-3}$, with a 95 % confidence interval of 133-255 $mg.m^{-3}$. Since the LC50 for SM without pretreatment is 160 $mg.m^{-3}$, with a 95 % confidence interval of 140-184 $mg.m^{-3}$, the LC50 calculated for SM after pretreatment with CIPE is not significantly higher. If we would be able to take the result observed for a challenge with 800 $mg.min.m^{-3}$ also into account, the LC50 after pretreatment with CIPE would certainly be lower than 225 $mg.m^{-3}$, and the 95-% confidence limits would extend even further. Therefore, it seems justified to conclude from these results that pretreatment with 5 mmol/kg CIPE (i.p.), at 1 min prior to a respiratory challenge with SM, does not provide a significant protection against SM.

III.13 PROTECTION OF THE SKIN OF HAIRLESS GUINEA PIGS AGAINST SULFUR MUSTARD VAPOR OR LIQUID BY TOPICAL SKIN PROTECTANTS

III.13.a. Relationship between Ct of exposure and concentration of DNA adducts

In order to test the efficacy of Topical Skin Protectants (TSP's) against a relevant challenge with SM vapor, he relationship between Ct of SM vapor exposure and skin damage in the hairless guinea pig was studied. Skin damage was assessed on the basis of the number of DNA adduct, measured via immunofluorescence microscopy, as well as by judging the redness of the skin according to Draize *et al.* (1946). Scoring the redness of the skin appeared to be very tedious, since the animal as whole turned red, which made a comparison with color of the non-exposed skin impossible After several attempts we abandoned the Draize-approach.
The results obtained with immunofluorescence microscopy are presented in Table 36 and Figure 44.

Table 36. Fluorescence intensity in arbitrary units as measured via immunofluorescence microscopy in skin biopts of hairless guinea pigs which were whole-body exposed to sulfur mustard vapor in air at various concentrations for 10 min.

Ct ($mg.min.m^{-3}$, ±'s.e.m., n=6)	Fluorescence intensity (a.u., ± s.e.m., n=6)
0	10,700 ± 180
95 ± 3	20,400 ± 1,200
560 ± 20	41,400 ± 1,900
1,560 ± 70	45,200 ± 1,500
3,500 ± 150	46,600 ± 1,800

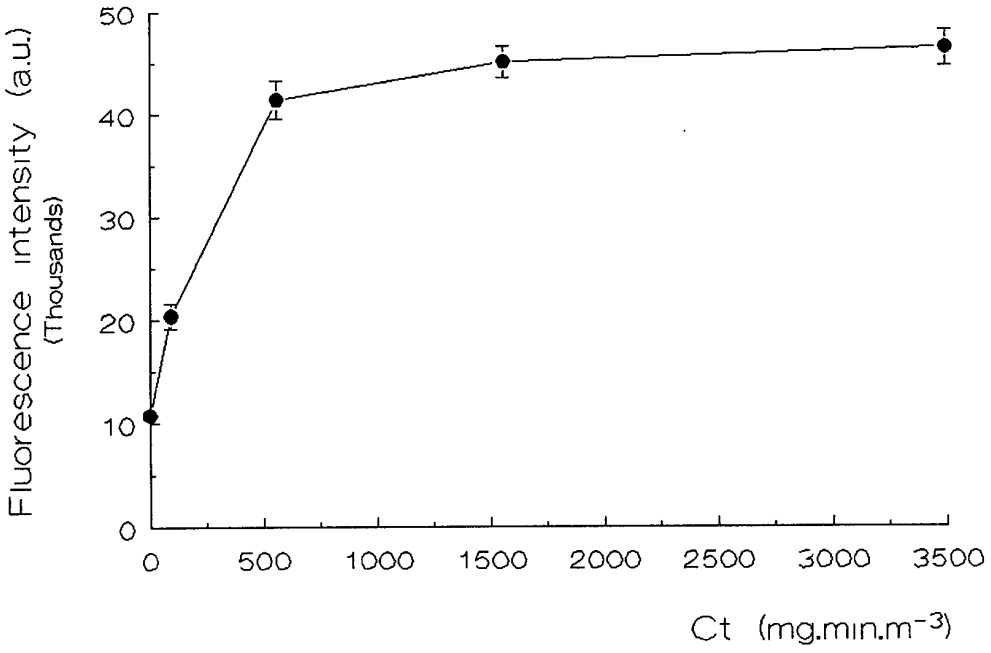

Figure 44. Fluorescence intensity (arbitrary units, ± s.e.m., n=6) versus the Ct ($mg.min.m^{-3}$) for whole-body exposure of hairless guinea pigs to sulfur mustard vapor in air, as measured via immunofluorescence microscopy.

The Ct values listed in Table 36 were calculated from the 'area under the curve' of the time-course of the concentration of SM vapor in air inside the exposure chamber during exposure, as measured by gas chromatography. The mean Ct values deviate to some extent from the target values that were chosen originally, i.e., 100, 500, 1,500 and 3,000 mg.min.m^{-3}. However, for the purpose of this part of the study this is not a problem. Obviously, there is a considerable background fluorescence encountered when applying immunofluorescence microscopy. Furthermore, the relationship between the Ct of exposure and the measured fluorescence intensity appears to be linear only in the Ct range below *ca.* 500 mg.min.m^{-3}. Above this Ct-value the fluorescence signal gradually starts to approach a limit value near 50,000 arbitrary units, A Ct of approximately 1,500 mg.min.m^{-3} was chosen as the challenge exposure for testing the TSP's. Should the TSP's provide an adequate protection, this should lead to an appreciable reduction in the number of DNA adducts in skin, and therefore in measured fluorescence.

In conjunction with these experiments, the DNA damage in the cornea of the animals exposed to Ct-values of *ca.* 500 and 3,000 mg.min.m^{-3}, was established. DNA could be easily isolated from the corneal epithelia. In three of the six animals whole-body exposed to a Ct of 3,500 mg.min.m^{-3} 7-SM-gua was detected with a mean concentration of 19 ± 4 adducts per 10^7 nucleotides (± s.e.m., n=3). According to the technicians who performed the exposures, the other three animals had their eyes closed during the exposure, which explains why no adducts could be detected in their corneal epithelia. Two of the six animals exposed to Ct of 560 mg.min.m^{-3} also had their eyes closed during exposure. Nevertheless, 7-SM-gua could not be detected in the DNA isolated from corneal epithelia of three of the remaining four animals. For one animal 0.13 adducts per 10^7 nucleotides was measured. The mean 7-SM-gua concentration for these four animals is therefore 0.03 ± 0.03 adducts/10^7 nucleotides (± s.e.m., n=4).

III.13.b. Protection by topical skin protectants against sulfur mustard vapor

The TSP's were applied on the skin of hairless guinea pigs, as described in section II.9. Figure 45 shows a photograph of a restrained hairless pig with the TSP's applied onto 8 of the 12 spots. The animals were exposed to SM vapor for *ca.* 10 min in the whole-body exposure chamber, at a Ct of approximately 1,500 mg.min.m^{-3}. The actual Ct was 1,740 ± 20 mg.min.m^{-3}. At 2 h after ending the exposure, skin biopts were taken for analysis by immunofluorescence microscopy. The results are presented in Table 37 and Figure 46.

The fluorescence intensities presented in Table 37 were measured with a Zeiss laser-scanning microscope, and cannot be compared with those listed in Table 36, which were measured a Leitz fluorescence microscope equipped with a CCD camera. This is not a problem, since both experiments are independent from each other.

Table 37. Fluorescence intensity in arbitrary units as measured via immunofluorescence microscopy in skin biopts of hairless guinea pigs whole-body exposed to sulfur mustard vapor in air at a Ct of 1,740 ± 20 mg.min.m^{-3} (10-min exposure) in the presence or absence of Topical Skin Protectants 1511 and 2701.

TSP	Fluorescence intensity (a.u., ± s.e.m., n=6)
None	137 ± 7
1511	74 ± 5
2701	64 ± 5
None, skin not exposed to SM	56 ± 6

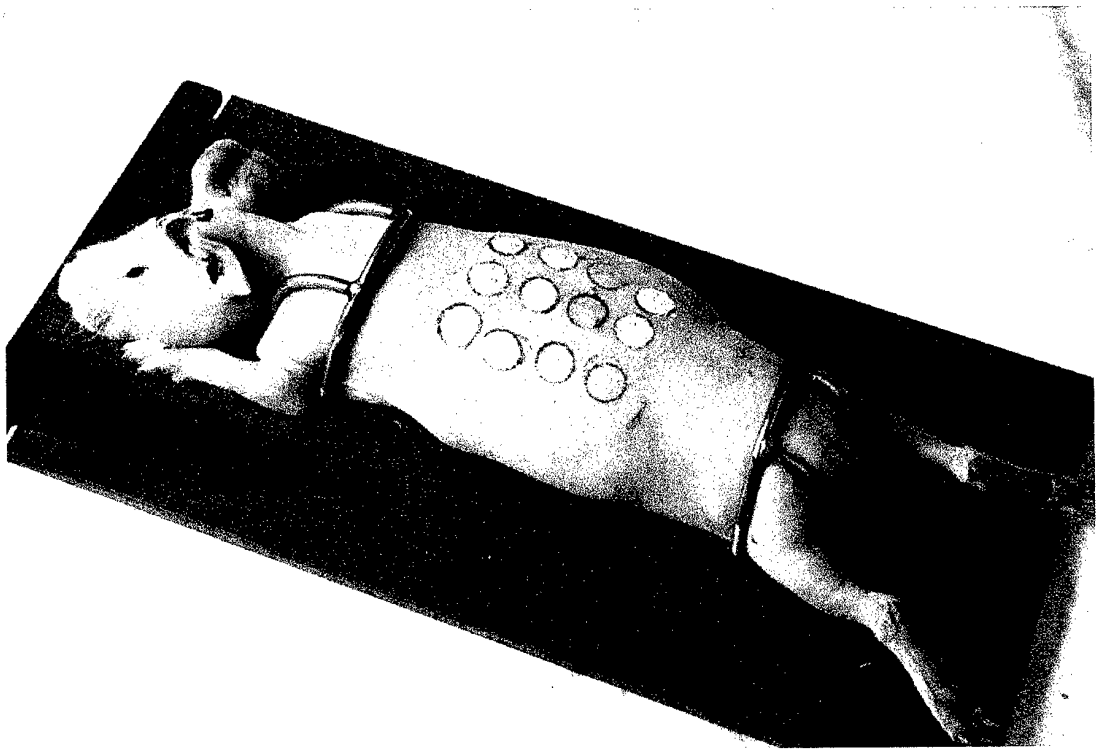

Figure 45. Photograph of a restrained hairless guinea pig prepared for testing Topical Skin Protectants against a challenge with SM liquid or vapor. The TSP's have been applied onto eight of the twelve spots marked with ink. The first two spots in the upper row are covered with TSP 2701, the next four spots with TSP 1511 followed by four unprotected spots and the two remaining TSP 2701 spots.

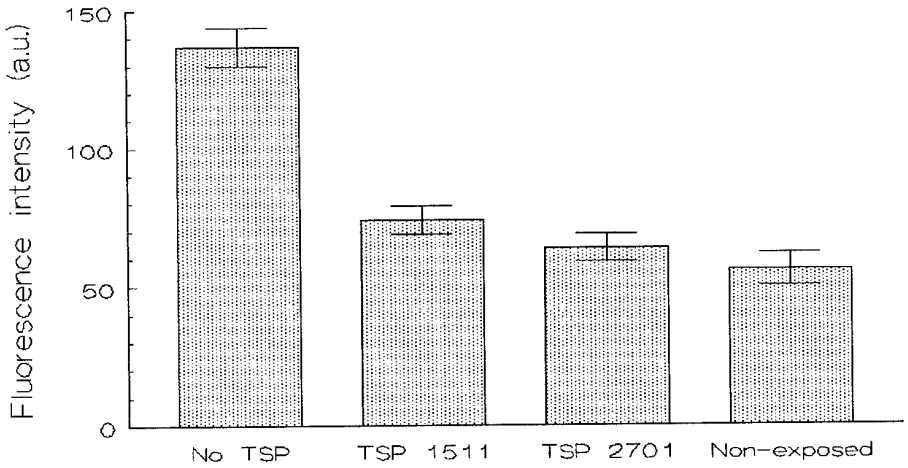

Figure 46. Fluorescence intensity (arbitrary units, ± s.e.m., n=6) measured via immunofluorescence microscopy for biopts of skin of hairless guinea pigs exposed to sulfur mustard vapor at a Ct of 1,740 ± 20 $mg.min.m^{-3}$ (10-min exposure) in the presence or absence of topical skin protectants 1511 and 2701.

The results presented in Table 37 and Figure 46 clearly show that the number of adducts in the skin resulting from exposure to a Ct of 1,740 ± 20 mg.min.m^{-3} is markedly reduced when TSP's are applied onto the skin. Statistical evaluatiuon of the data with the Student's t-test at the 0.05 probability level indicated a significantly higher fluorescence for the exposed skin which was not covered with a TSP, in comparison with non-exposed skin or exposed skin covered with TSP 1511 or 2701. There are no significant differences between the fluorescence signals of the non-exposed skin, or the exposed skin samples covered with either of the two TSP's. These results suggest that the two TSP's provide an equally high protection against a challenge with SM vapor at a Ct of 1,740 ± 20 mg.min.m^{-3}.

III.13.c. Protection by topical skin protectants against liquid sulfur mustard

TSP's were applied onto the skin of hairless guinea pigs, as described in section II.9. The skin spots were subsequently contaminated with liquid SM, and decontaminated 2 h later. On most of the skin spots covered with a TSP, a droplet of SM was still clearly visible. On the skin sites that were not protected with a TSP the agent was no longer visible. At 2 h after decontamination, skin biopts were taken for analysis by immunofluorescence microscopy. The results are presented in Table 38 and Figure 47.

Table 38. Fluorescence intensity in arbitrary units as measured via immunofluorescence microscopy in biopts of skin sites of hairless guinea pigs exposed for 2 h to liquid sulfur mustard, in the presence or absence of Topical Skin Protectants 1511 and 2701.

TSP	Fluorescence intensity (a.u., ± s.e.m., n=6)
None	138 ± 12
1511	65 ± 13
2701	38 ± 6
None, skin not exposed to SM	9 ± 1

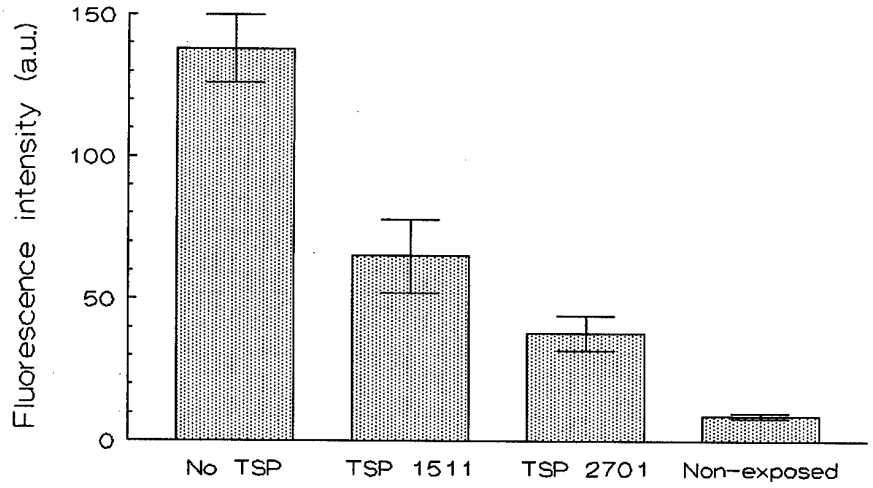

Figure 47. Fluorescence intensity (arbitrary units, ± s.e.m., n=6) measured via immunofluorescence miscrocopy for biopts of skin of hairless guinea pigs exposed for 2 h to liquid sulfur mustard, in the presence or absence of topical skin protectants 1511 and 2701.

The results presented in Table 38 and Figure 47 clearly show that the number of adducts in the skin resulting from exposure liquid SM is markedly reduced when TSP's are applied onto the skin. Statistical evaluatiuon of the data with the Student's t-test at the 0.05 probability level indicated a significantly higher fluorescence for the exposed skin which was not covered with a TSP, in comparison with non-exposed skin or exposed skin covered with TSP 1511 or 2701. Furthermore, the fluorescence measured for the skin biopts covered with TSP 1511 or 2701 is significantly higher than that of the non-exposed skin. There are no significant differences between the fluorescence signals measured for biopts covered with the two TSP's. These results suggest that the two TSP's protect equally well against a challenge with liquid SM.

IV. DISCUSSION

Analytical procedures

The analytical procedures developed for SM appeared to be very convenient. Gas chromatography with mass-spectrometric detection (GC-MS) under semi-single ion conditions provides for a highly selective and sensitive method of analysis. An additional advantage of MS-detection is the possibility to identify the peak that is being quantified. This option was used frequently throughout this study. The automated thermodesorption injection (TDAS) increases the daily sample-throughput and allows large volume injection with concomitant on-line clean-up of the extract, thus limiting contamination of the gas chromatography columns and improving the reliability of the analysis. Unfortunately, our TDAS suffered from a considerable down-time due to wear and tear. Nevertheless, the workload for the technicians was reduced considerably by using the TDAS, which was convenient in view of the large number of samples which had to be analyzed. For most of the samples analyzed, the detection limit of the TDAS-GC-MS configuration of 5 pg/ml was well below the concentration of SM that were actually measured in the samples, which is beneficial for the reliability of the produced data.

The liquid-liquid extraction procedure with ethyl acetate is straightforward, reproducible and provides for a high recovery. As was anticipated, D_8-SM is a suitable internal standard for the bioanalysis of SM.

Gas chromatography with pulsed-flame photometric detection (GC-PFPD) (Amirav and Jing, 1995) appears to be an attractive alternative for GC-MS analysis, in particular when combined with thermal cold trap injection (TCT). Although the detection limit for TCT-GC-PFPD is *ca.* 25-fold higher than for TDAS-GC-MS, i.e., *ca.* 125 pg per ml of blood or g of tissue, the majority of the samples could easily be measured with the former technique. An advantage of PFPD is that it combines the selectivity of flame-photometric detection with the sensitivity of electron capture detection. The PFPD appeared to be a highly stable and reliable detector. The TCT-GC-PFPD configuration is about fourfold cheaper than the TDAS-GC-MS configuration.

The immunoslotblot (ISB) assay for the major adduct of SM to DNA, i.e., 7-SM-gua, was already developed by Dr. Van der Schans within the context of USAMRDC Grant DAMD17-88-Z-8022 (Benschop, 1991), and has been successfully applied ever since. During the current study, the detection limit of the ISB assay has been further improved with a factor of 5 by modifying the DNA isolation procedure, the binding of the isolated DNA to the nitrocellulose filter and by measuring the chemiluminescence by means of a luminometer instead of via the blackening of a photographic film. In the course of the study, we encountered samples to which the ISB assay could not be applied, apparently due to a very large extent of DNA damage, associated with cross-linking. DNA could not be isolated from these samples. This causes some practical problems, but also raises questions with respect to the reliability of the results of the ISB assay. One may wonder whether the ISB assay underestimates the actual DNA damage, since apparently only DNA with a relatively low number of adducts can be isolated. It is difficult to verify whether the ISB assay produces the correct results. For the time being, the amount of DNA that could be isolated from the samples was considered, as a guidance to judge whether the extent of DNA damage was too high to be analyzed with the ISB assay.

For samples with very high numbers of DNA adducts, another technique developed in the context of the aforementioned Grant was applied, i.e., immunofluorescence microscopy (IFM). This technique suffers less from cross-linking, since the DNA does not have to be isolated from the tissue, for quantification of the number of adducts. However, IFM is a more expensive technique than ISB, and the quantification of the number of adducts with IFM is less reliable than with the ISB assay.

In recent years, we have developed alternative methods to analyze 7-SM-gua, i.e., by using high-performance liquid chromatography with electrochemical detection (HPLC-ElCD) or tandem MS detection (HPLC-MS-MS). These methods have been used succesfully to quantify DNA

damage in pig's ear epidermis resulting from exposure to SM vapor (Langenberg *et al.* 1995). The sensitivities of HPLC-ElCD or HPLC-MS-MS for 7-SM-gua are comparable to that of the ISB assay. The selectivity of the HPLC-ElCD method is somewhat less than that of the ISB or HPLC-MS-MS. Furthermore, a drawback of the HPLC-based methods is that they require considerably larger samples than the ISB. It can be concluded that for the time being the HPLC-based methods have no real advantages over the ISB procedure.
Since the analysis of 7-SM-gua via ISB, HPLC-ElCD or HPLC-MS-MS requires the isolation of DNA, all techniques appear to fail for samples with very high extents of DNA damage. In such cases immunofluorescence microscopy (IFM) appeared to be the only suitable alternative. The IFM procedure was applied successfully in the current study, i.e., to quantify DNA damage in skin of hairless guinea pigs exposed to SM as vapor or liquid.

The hairless guinea pig as an animal model in toxicokinetic studies of SM
The hairless guinea pig has been advocated to be a suitable small animal model for studies on (per)cutaneous toxicology of SM (Mershon *et al.* 1990, Papirmeister *et al.* 1991). The epidermis of the hairless guinea pig is thicker than in most other laboratory animals and therefore more similar to the human epidermis. Nevertheless, the cell layers in its epidermis are thinner than those in man. Furthermore, the stratum corneum of the hairless guinea pig is considerably thicker than that in man. This has to be taken into account when trying to extrapolate the results obtained in hairless guinea pigs to man. In this respect the pig would be a better model for man, since its skin resembles that of man more closely. However, the pig is far from a small laboratory animal. Its use in e.g. respiratory or percutaneous studies will be accompanied by technical difficulties.
A problem associated with the use of the hairless guinea pig as an animal model is its limited availability. In 1994, the hairless guinea pig colony of Charles River in the USA was lost due to an infection. A new colony was started, using animals from Charles River Germany, but up to now the availability of animals in the US is still problematic. In Europe the situation is somewhat better, albeit that the substantial extension of the current study is mainly due the inavailability of a sufficient number of animals in the desired weight range when we needed them. These problems arose when Charles River decided to move its hairless guinea pig breeding facility from Germany to Hungary. From that moment the breeding capacity was reduced, in view of the limited market. Furthermore, we noticed that the microbiological status of the animals deteriorated gradually. The animals appeared to be in a good condition but a fraction of the population carried virusses such as Reo-3, which one prefers to keep outside the animal facilities. Unfortunately, Charles River refused to sanitize this colony. About a year ago we learned that the Swiss company BRL (represented in The Netherlands by Harlan), which also supplies hairless guinea pigs. The BRL hairless guinea pig is essentially the same as the animal from Charles River, since both originate from the mutant that was identified by the Institute Armand Frappier (IAF) in 1978. The animals obtained from BRL have an excellent microbiological status, are much more lively than those of Charles River Hungary, and are available in sufficient quantities. Unfortunately, we did not endeavour to switch between animal suppliers during the course of this study, since it could not be ascertained that the results would be comparable. Consequently, this study was performed with animals from Charles River, despite the fact that the situation became exponentially worse with time. In view of our positive experiences with BRL, it is still possible to use the hairless guinea pig in future studies.

Intravenous toxicokinetics
The intravenous 96-h LD50 of SM, i.e., 8.2 mg/kg in the hairless guinea pig, is relatively high in comparison with that rats (3.8 mg/kg, 14-day LD50, Maisonneuve *et al.*, 1993; 0.7 mg/kg, 15-day LD50, Anslow *et al.*, 1948) and rabbits (2.7 mg/kg, 14-day LD50, Anslow *et al.*, 1948) but is comparable to the value reported for mice (8.6 mg/kg, 15-day LD50, Anslow *et al.*, 1948).
The concentration-time courses of SM after intravenous doses of 8.2 and 2.46 mg/kg, corresponding with 1 and 0.3 LD50, respectively, are shown in Figures 48 and 48a.

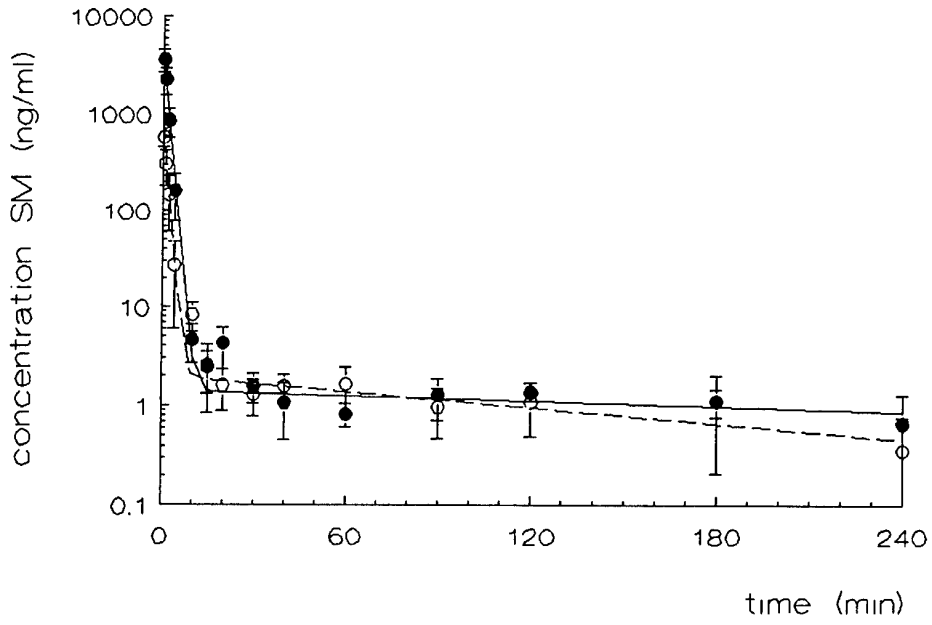

Figure 48. Mean concentration-time courses of SM (± s.e.m., n=6) in blood of anesthetized male hairless guinea pigs after i.v. administration of doses of SM corresponding with 1 (•) and 0.3 (o) LD50.

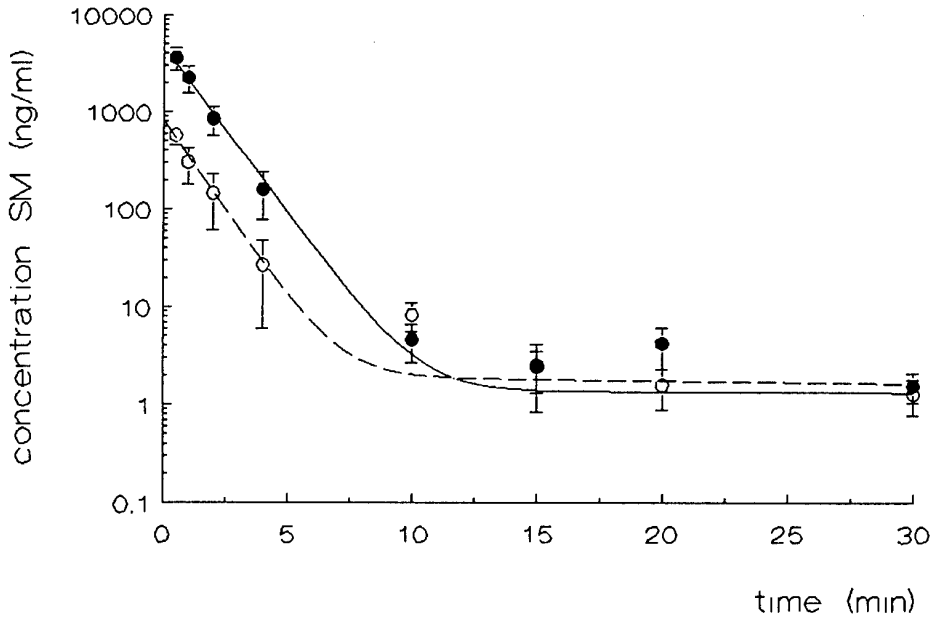

Figure 48a. The first 30 min of Figure 48 on an expanded time-scale.

The intravenous toxicokinetics of SM in the hairless guinea pig are characterized by a very rapid distribution phase and a slow elimination phase, and can be adequately described with a bi-exponential equation. The time-courses for both doses are very similar. The toxicokinetic parameters derived from these two concentration-time courses are presented in Table 39. For the purpose of comparison, toxicokinetic parameters for an intravenous bolus administration of 10 mg SM/kg to rats, calculated from the coefficients and exponents reported by Maisonneuve *et al.*(1993) as well those for C(-)P(-)-soman (Benschop and Van Helden, 1993) after intravenous bolus administration of a dose corresponding with 0.8 LD50 are incorporated into this table.

Table 39. Toxicokinetic parameters[a] for SM in the hairless guinea pig for intravenous bolus administration of doses corresponding with 0.3 and 1 LD50 (96-h). Toxicokinetic parameters for SM in the rat and for C(-)P(-)-soman in the guinea pig are presented for comparison.

Parameter	SM, 2.46 mg/kg, 0.3 LD50 i.v., hairless guinea pig	SM, 8.2 mg/kg, 1 LD50 i.v., hairless guinea pig	SM, 10 mg/kg, 3 LD50 i.v., rat[b]	C(-)P(-)-soman, 4.95 µg/kg[c], 0.8 LD50 i.v., guinea pig[d]
Number of exponents	2	2	2	2
A ($ng.ml^{-1}$)	826	4665	1293	3.8
a ($ng.ml^{-1}$)	1.97	1.41	45.8	0.80
B (min^{-1})	0.85	0.78	0.12	0.95
b (min^{-1})	0.0061	0.002	0.0032	0.12
AUC ($ng.min.ml^{-1}$)	1294	6686	25088	10.6
C_0 ($ng.ml^{-1}$)	828	4666	1339	4.6
$k_{1,2}$ (min^{-1})	0.209	0.082	0.063	0.37
k_{el} (min^{-1})	0.64	0.70	0.053	0.44
$k_{2,1}$(min^{-1})	0.0081	0.0022	0.0072	0.26
$t_{½,dis}$ (min)	0.81	0.89	5.8	0.73
$t_{½,el}$ (min)	114	347	216	5.78
V_1 ($l.kg^{-1}$)	2.97	1.76	7.47	1.08
V_{dss} ($l.kg^{-1}$)	79.55	66.07	72.8	2.62
Cl ($l.min^{-1}.kg^{-1}$)	1.90	1.23	0.40	0.47
MRT (min)	41.85	53.87	181	5.6

[a] The concentration of sulfur mustard at time t is described by: $[SM] = A*e^{-at} + B*e^{-bt}$

[b] Data calculated from Maisonneuve *et al.* (1993)

[c] Administered dose of C(±)P(±)-soman: 22 µg/kg

[d] Data calculated from Benschop and Van Helden (1993)

Abbreviations used: AUC, area under the curve; C_0, retrapolated concentration in the central compartment at time 0; $k_{1,2}$, rate constant of transfer from compartment 1 to compartment 2; k_{el}, rate constant of elimination; $k_{2,1}$, rate constant of transfer from compartment 2 to compartment 1; $t_{½,dis}$, distribution half-life; $t_{½,el}$, terminal half-life; V_1, volume of the central compartment; V_{dss}, volume of distribution under steady-state; Cl, total body clearance; MRT, mean residence time.

The half-life of the first phase in the intravenous toxicokinetics of SM in the hairless guinea pig is less than 1 min, which is in the same order of magnitude as measured for C(-)P(-)-soman in the Dunkin-Hartley guinea pig, but about 7-fold more rapid than for SM in the rat at a dose corresponding with 3 LD50. Such a short half-life indicates either a rapid elimination process, for instance binding (as in the case of soman), hydrolysis, or partitioning to the tissues. The data on the concentrations of intact SM in tissues clearly indicate a substantial partitioning of SM from the blood into the tissues. The high ratio of $k_{1,2}$ over $k_{2,1}$ is in agreement with a rapid 'loading' of the tissues with SM. Furthermore, the half-life of the first toxicokinetic phase is at

least 5-fold shorter than the reported half-life of hydrolysis of SM in aqueous solution (Bartlett and Swain, 1949). Consequently, it seems reasonable to designate the first phase in the toxicokinetics as a distribution phase.
From time-point 3 min after intravenous administration of a dose corresponding with 1 LD50, the concentrations of intact SM in lung, spleen, liver and bone marrow begin to exceed the concentration in blood (*cf.* Table 9 and Figures 12 and 13). Initially, the highest tissue concentration is found in the lung. This is not surprising, since after administration of SM into the jugular vein, the lung is first served. At 6 h after administration of a dose corresponding with 1 LD50, the concentrations of SM in spleen, liver and bone marrow are considerably higher than those in blood, whereas the concentration of SM in the lung is about equal to that in blood. The same pattern is observed for a dose corresponding with 0.3 LD50, albeit that the difference between tissue and blood concentrations is less pronounced. Unfortunately, the analyses of intact SM in fat tissue have failed, which was anticipated to be one the tissues of major importance with respect to the distribution of SM. Obviously, the long terminal half-life of SM in blood is the result of redistribution from the tissues into the blood. The terminal half-lives calculated for doses corresponding with 0.3 and 1 LD50 are reasonably comparable with the value reported for the rat by Maisonneuve *et al.* (1993).
Comparison of the AUC values calculated for administration of doses corresponding with 1 and 0.3 LD50 indicates non-linearity of the toxicokinetics with the dose. Usually, non-linear toxicokinetics with dose are associated with saturable metabolic pathways. At this point it is not clear which process is responsible for the non-linear toxicokinetics in the hairless guinea pig. Tentative explanations are covalent binding of SM to e.g., glutathione and albumine.
After correction for the difference in administered dose per kg bodyweight, the AUC in the rat is 3-fold higher than in the hairless guinea pig. This could be the result of interspecies variation, but also of non-linearity with dose. Since the AUC and the clearance (Cl) are related parameters, the total body clearance of SM in the hairless guinea pig is *ca.* 3-fold higher than in the rat.
The rate constants of transfer between compartments 1 and 2, i.e., $k_{1,2}$ and $k_{2,1}$, are comparable for the two species, whereas the elimination rate constant, k_{el}, is 15-fold higher in the hairless guinea pig than in the rat. Marked differences are observed between the values calculated for the volume of the central compartment (V_1) and the volume of distribution under steady-state (V_{dss}) for the two species. Furthermore, there is a 25-fold difference in the mean residence time (MRT). This parameter represents the mean lifetime expectancy of each SM molecule in the body.

The concentrations of intact SM in tissues after intravenous administration of a dose corresponding with 0.3 LD50 (*cf.* Table 14) are more than 3.3-fold lower than those measured after a dose corresponding with 1 LD50 (*cf.* Table 9), underscoring the abovementioned non-linearity of the toxicokinetics with dose. In Figure 49 the time-courses of the concentrations intact SM in liver are compared for the two intravenous doses.

After administration of a dose of SM corresponding with 1 LD50, 7-SM-gua was detected in all tissues studied, i.e., blood, lung, spleen, bone marrow, liver and small intestine, after intravenous administration (*cf.* Table 10). Large differences in adduct concentrations are observed between the various tissues as well as in the time courses of the adduct concentrations (*cf.* Figures 14-16). Interestingly, the time-courses of 7-SM-gua in various tissues follow the general pattern of those of intact SM in these tissues. By far the highest concentration of 7-SM-gua is observed in lung tissue. After administration of a dose corresponding with 1 LD50, the adduct level in lung is already high at 3 min after administration and remains high up to 48 h after administration. It is remarkable that the adduct level in blood, as early as 3 min after intravenous administration, is almost 30-fold lower than in the lung. At 48 h after administration, the adduct level in blood is *ca.* 1 per 10^7 nucleotides, which is still *ca.* 20-fold lower than in the lung. This observation can at least partly be explained from the extensive partitioning of SM from the blood into the lung. In addition, a higher reactivity of SM with

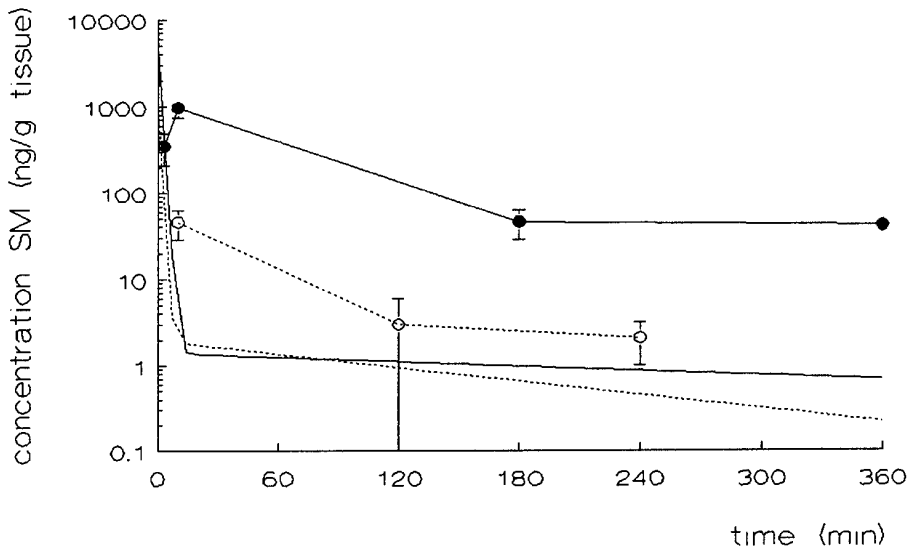

Figure 49. Concentration (ng/g, ± s.e.m.) of intact SM in liver of hairless guinea pigs at various time points after intravenous administration of 8.2 (●) or 2.46 (○) mg/kg, which corresponds with 1 and 0.3 LD50 (96-h), respectively. The concentration-time profiles of SM in blood are also presented for 1 (solid line) and 0.3 (dotted line) LD50.

DNA in lung cells than with DNA in white blood cells cannot be excluded. However, at this point we have no data to support this hypothesis.
In the spleen, the concentration of 7-SM-gua increased in the period up to 3 h after administration. After 24 h the number of adducts has decreased to *ca.* 1 per10^7 nucleotides. In bone marrow the adduct level showed some variation in the first 6 h, after which the concentration of 7-SM-gua gradually decreased.
The adduct level in the liver appeared to be maximal at 3 h after administration. The level had decreased at 6 h, whereas the adducts had nearly disappeared at 24 h. In the small intestine a fast accumulation of adducts during the first 10 min after administration was observed. The adduct level subsequently decreased, but remained at a steady level of about 2 adducts per 10^7 nucleotides up to 48 h after administration.
The concentrations of 7-SM-gua in tissues after i.v. administration of a dose corresponding with 0.3 LD50 (*cf.* Table 15) were more than 3.3-fold lower than those measured for 1 LD50, underscoring the non-linearity of the toxicokinetics with the dose. At 10 min after administration of 0.3 LD50 SM, the highest adduct levels were found in blood (5 adducts per 10^7 nucleotides) and the lung (*ca.* 3 adducts per 10^7 nucleotides), whereas in spleen and small intestine the levels were *ca.* 0.5 adducts per 10^7 nucleotides. In bone marrow no adducts were detectable at 10 min after administration of this dose. At 48 h after administration the adduct had largely dissappeared from lung, spleen, bone marrow and small intestine, whereas the level in blood was only slightly lower. Interestingly, the concentration of 7-SM-gua in lung does not reach such extreme values as was observed after administration of a dose corresponding with 1 LD50. This finding is in agreement with the relatively low concentration of intact SM in lung tissue after intravenous administration of a dose corresponding with 0.3 LD50. However, the concentration of 7-SM-gua in blood is in the same order of magnitude for both doses shortly after administration of SM. This seems to indicate that a substantial amount of SM will bind covalently immediately in the blood, which is a saturable process.
The time courses of 7-SM-gua for the two intravenous doses are compared for lung and blood in Figures 50 and 51, respectively.

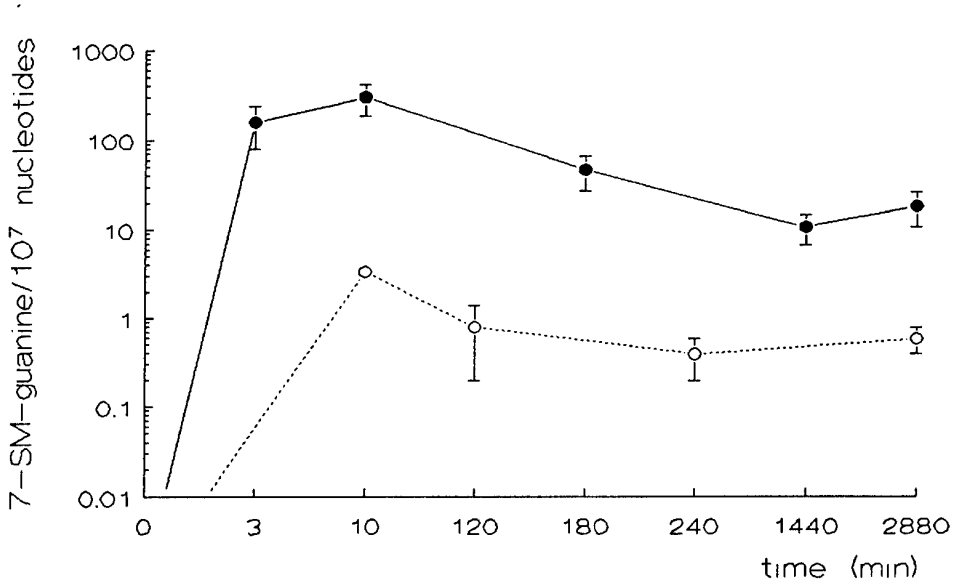

Figure 50. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides, ± s.e.m., in DNA of the lung of hairless guinea pigs at various time points after intravenous administration of doses corresponding with 1 (●) and 0.3 (○) LD50 (96-h). The labels along the X-axis represent the time points (in min) after administration.

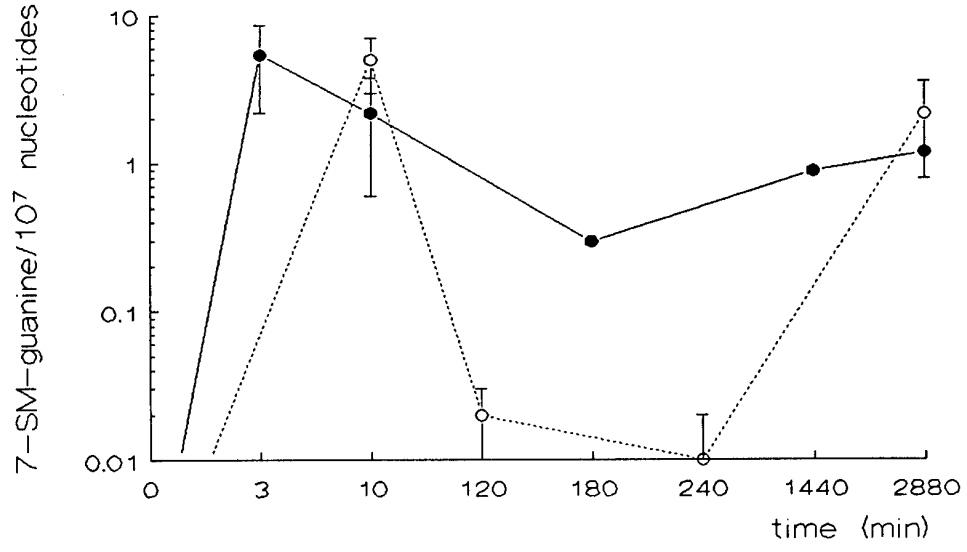

Figure 51. Concentration of 7-SM-gua, expressed as number of adducts per 10^7 nucleotides, ± s.e.m., in DNA of the blood of hairless guinea pigs at various time points after intravenous administration of doses corresponding with 1 (●) and 0.3 (○) LD50 (96-h). The labels along the X-axis represent the time points (in min) after administration.

The toxicokinetic parameters for C(-)P(-)-soman after i.v. administration of a dose corresponding with 0.8 LD50 C(±)P(±)-soman in the atropinized guinea pig were calculated from the coefficients and exponents of the biexponential equation as reported previously (Benschop and Van Helden, 1993). Although soman and SM differ very strongly in their (bio)chemistry and toxicology, it is interesting to see how their toxicokinetics compare. The distribution half-lives of SM (1 LD50) and C(-)P(-)-soman are nearly the same, whereas the elimination half-life of C(-)P(-)-soman is *ca.* 60-fold shorter than that of SM. The AUC of C(-)P(-)-soman is nearly 600-fold lower than that of SM, which can explained by the 1600-fold lower dose of C(-)P(-)-soman in comparison with SM. The transfer rate constants between compartments 1 and 2 are higher for C(-)P(-)-soman than for SM , whereas the elimination rate constants are of the same order of magnitude. The volumes of the central compartment are nearly the same for SM and C(-)P(-)-soman, whereas the steady-state distribution volume of SM is nearly 3-fold larger than that of C(-)P(-)-soman, despite the fact that the n-octanol/water partition coefficient for SM (log P_{ow} 1.37) is lower than that of C(±)P(±)-soman (log P_{ow} 1.78) (FOA, 1992). The most plausible explanation for the observed extensive partitioning of SM to the tisses is that SM apparently degrades much slower when distributed into the tissues than soman.
The total body clearance of SM is about 2.5-fold higher than that of C(-)P(-)-soman. The mean residence time of SM is nearly one order of magnitude higher than that of C(-)P(-)-soman.

The intravenous toxicokinetics of SM in hairless guinea pig seem to be in reasonable agreement with that reported by Maisonneuve et al. (1993) for the rat. However, we recently obtained indications that an important interspecies variation may exist. Preliminary data on the intravenous toxicokinetics of SM in the marmoset for a dose of 8.2 mg/kg showed that the toxicokinetics of SM in blood of this non-human primate are best described with a tri-exponential equation (Langenberg *et al.*, 1997b). The first phase was *ca.* 2.5-fold slower than in the hairless guinea pig, whereas the rate constant of the third phase was comparable to that of the elimination phase in the hairless guinea pig. The AUC of this dose in the marmoset was twofold higher than in the hairless guinea pig. Up to one hour after intravenous administration, the concentration of SM in blood of the marmoset was nearly one order of magnitude higher than in the blood of the hairless guinea pig. Interestingly, the 7-SM-gua concentrations in the tissues of the marmoset were consistently lower than in the hairless guinea pig. These findings taken together suggest less partitioning of SM from the blood into the tissues in the marmoset. If that is the case, the concentrations of intact SM in the marmoset tissues should also be lower. Unfortunately,, these concentrations have not been measured yet.

The strategy for (pre)treatment of intoxication with SM would benefit from a definition of the minimal toxicologically relevant level of agent in blood and/or tissues. For the nerve agent soman the toxicologically relevant level was defined as the AUC which would be able to (re)inhibit the minimum essential concentration of acetylcholinesterase with a half-life of 1 h (Benschop and De Jong, Final Report for Grant No. DAMD17-87-G-7015,1990). The blood concentration of C(±)P(-)-soman which corresponded with this threshold value was calculated to be 150 pM (*ca.* 30 pg/ml).
For soman the calculation of lowest concentration of toxicological relevance is relatively simple, since the toxic effect is well defined, which is not the case for SM. Reduction of the viability of cells within 24 h of exposure can be used as a criterion for defining the lowest concentration of toxicological relevance. Exposure of cells to 75 µM (*ca.* 12 µg/ml) of SM reduces cell viability within 24 h, whereas 50 µM has no apparent effect (Mol, 1996). This concentration is more than 2-fold higher than the retrapolated blood concentration of SM at time 0 after i.v. administration of 8.2 mg/kg SM. Since this dose corresponds with 1 LD50, it seems unlikely that 75 µM is a realistic value for the lowest level with toxicological relevance. It is also known that DNA adducts can be detected after exposure of white blood cells to 10 nM (*ca.* 1.6 ng/ml) SM for 1 h. This adduct formation can be considered to be an adverse effect,

although the correlation between adduct formation and cytotoxicity is unclear, and some repair of the DNA damage may occur. A concentration of 1.6 ng of SM per ml of blood will already be reached at *ca.* 18 min after i.v. administration of 1 LD50 SM. However, our measurements show that the concentrations of SM in tissues exceed the concentration in blood for a considerable period of time after administration. This underscores the need to follow the toxicokinetics of SM in blood and tissues for several hours instead of 18 min, until the concentrations of intact SM in relevant compartments can no longer induce toxic effects.
Obviously, more insight into the concentrations of SM which cause adverse effects in tissues is needed. Whether 10 nM is a realistic estimation of the lowest level of toxicological relevance should be a topic for further investigation and discussion. In such a discussion the relative importance of skin, respiratory tract, and systemic damage will have to be taken into consideration.
Since SM is designated as a primary carcinogenic compound, one could also advocate to define the toxicologically relevant concentration as the lowest concentration which is carcinogenic. However, such a concentration is unknown for SM. The most conservative approaches to defining carcinogenic concentrations are based on the one-hit model, assuming that even 1 molecule of a carcinogenic compound can cause cancer (Cornfield, 1977). Obviously, our method is not suitable for detection of 1 molecule SM in the body of the hairless guinea pig.

Inhalation toxicokinetics
Initially, we were rather surprised that the concentrations of SM in blood were below the detection limit during and after nose-only exposure to 1 LCt50. However, the observation that no 7-SM-gua could be detected in spleen, bone marrow and small intestine, while rather low concentrations of 7-SM-gua were measured in the lung at 10 min and 48 h after ending the exposure is in agreement with this outcome. Most clarifying is the distribution of 7-SM-gua within the respiratory tract at 4 h after ending the 5-min nose-only exposure as shown in Figure 26. Adduct formation occurs mainly in the larynx and trachea, whereas almost no SM appears to have reached the lung. It is evident that SM causes lethal damage in the respiratory tract without a systemic uptake to such an extent that systemically lethal concentrations can build up. This mechanism stands in contrast with that for nerve agents, which are absorbed in the (upper) respiratory tract to cause mainly systemic toxicity.
Cameron *et al.* (1946) have also observed that hardly any SM reaches the lung in rabbits, based on measurements of the concentration of SM in air sampled from the trachea via a cannula. They hypothesized that absorption of lethal doses of SM occurs via the nasal mucosa. However, since we could not detect SM in blood the present results seem to suggest that the respiratory toxicity of SM is overwhelmingly of a local rather than of a systemic nature. Obviously, in species with a less complex nasal system such as man, a larger fraction of the inhaled SM may reach the lung. This may lead to lung damage by direct reaction of SM with the lung tissues and/or indirectly via systemic uptake. It would be worthwhile to study the inhalation toxicokinetics of SM in for instance the marmoset monkey, and compare the results with those obtained for the hairless guinea pig.
The mean concentration-time course of SM in blood during and after nose-only exposure to 3 LCt50 SM, as shown in Figure 28, is rather capricious. So far, we have not succeeded in obtaining a reasonable mathematic description for these data. The absorption phase can be reasonably well described with a mono-exponential equation, but a meaningful two-exponential fit of the distribution and elimination phases has not yet been obtained. In fact, the course of the concentration SM with time seems to indicate that at least two absorption phases are present. One of these phases is relatively rapid, and is reflected in the rise of the concentration of SM in blood during the 8-min nose-only exposure. The sharp fall of the concentration of SM in blood after ending the exposure may be explained from the very rapid distribution, as observed in the intravenous toxicokinetic experiments. The second absorption phase is much slower than the first one, and is responsible for the rise in blood concentration after time point 20 min. Such a second absorption phase can be envisaged by assuming that SM deposits on the epithelia in the

upper respiratory, from which it is absorbed relatively slowly. The area under the curve (AUC), as calculated from 0 to 240 min with the trapezoidal approach, is 320 ng.min.ml^{-1}, which is 25-fold lower than the AUC (time 0 to ∞) calculated for 1 LD50 SM (i.v.).
A complicating factor with respect to the toxicokinetics during and after nose-only exposure to 3 LCt50 is our observation that in half of the blood samples no SM could be detected, including all samples taken at 15 and 20 min (which is why the curve is interrupted between time points 10 and 30 min in Figure 28). Surprisingly, SM was detected again in half of the samples taken at ≥ 60 min. In two of the twelve animals used to obtain n=6 for each time point SM could not be detected in any of the blood samples.
Intact SM could be detected in tissues at 10 min after ending the 8-min nose-only exposure to 3 LCt50 SM at concentrations comparable to those measured at 10 min after intravenous administration of a dose corresponding with 0.3 LD50 (*cf.* Tables 24 and 14). At *ca.* 4 h after ending the exposure, the concentrations of SM in tissues have decreased considerably, and are below the detection limit in lung and bone marrow.
The data in Table 25 indicate that the concentrations of 7-SM-gua in tissues are very low at 10 min and 2 and 4 h after nose-only exposure to 3 LCt50 of SM. The highest concentration was observed in the lung, at a level comparable with that observed after i.v. administration of 0.3 LD50.
Obviously, the inhalation toxicokinetics of SM needs further investigation.

Percutaneous toxicokinetics
It seems that the percutaneous absorption of SM proceeds fairly rapidly, since already at 10 min after starting the exposure to a vapor concentration of *ca.* 250 mg.m^{-3} SM concentrations in blood of *ca.* 1 ng/ml were measured. The concentration increases during the 40-min exposure period to *ca.* 12 ng/ml , after which it starts to decrease. At time point 240 min, the concentration is still as high as 0.9 ng/ml. Such a fairly high percutaneous absorption rate was anticipated in view the reported rapid cutaneous uptake of SM vapor by the hairless guinea pig of 2 μg.cm^{-2}.min^{-1} (Logan *et al.*, 1996).
There is a striking similarity between the concentration-time profiles of SM in blood for percutaneous and respiratory exposure. In Figures 52 and 52a the concentration-time profiles of SM in blood during and after nose-only exposure to a Ct of 2,400 mg.min.m^{-3} as well as percutaneous exposure to a Ct of 10,000 mg.min.m^{-3} are shown. As for the inhalation toxicokinetics, the percutaneous toxicokinetics of SM in the hairless guinea pig appear to be characterized by the existence of two absorption phases, one relatively rapid and one relatively slow. The slow absorption phase may be due to absorption of SM into the blood from skin depots which were 'loaded' during the exposure. As for the inhalation toxicokinetics, a 'dip' in the concentration of SM in blood is observed after ending the exposure. As stated above, this may be due to the rapid distribution of SM from the blood to the tissue as observed for the intravenous toxicokinetics.
After ending the 8-min nose-only exposure, the animals have been exposed to a Ct of *ca.* 2,400 mg.min.m^{-3}. At time-point 10 min of the percutaneous exposure, the animals have been exposed to a Ct of 2,500 mg.min.m^{-3}. Upon comparison of the concentration-time profiles in blood for these two exposures, for instance in Figure 52a, it is clear that the concentration build-up of SM in blood proceeds more rapidly for the nose-only exposure, suggesting that absorption via the respiratory route is more rapid than via the percutaneous route.
Interestingly, the maximum SM concentration in blood observed for percutaneous exposure to 10,000 mg.min.m^{-3} is approximately 3-fold higher than that for nose-only exposure to 2,400 mg.min.m^{-3}.
From the percutaneous toxicokinetic experiments it can be concluded that systemic intoxication from SM is more likely to occur from a percutaneous exposure than from a respiratory exposure, at least in the hairless guinea pig.
Concentrations of intact SM in tissues after percutaneous exposure appear to be relatively high, generally even higher than observed after intravenous administration of a dose corresponding

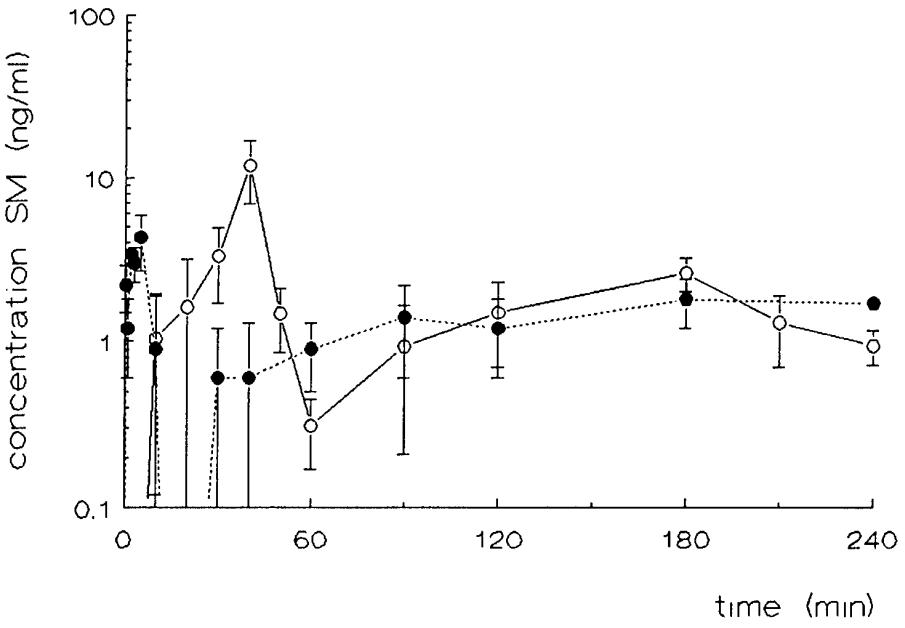

Figure 52. Mean concentration-time courses (ng/ml, ± s.e.m.) of sulfur mustard in blood of anesthetized, restrained hairless guinea pigs during and after 8-min nose-only exposure to a Ct of 2,400 mg.min.m^{-3} (●), and 45-min percurtaneous exposure to a Ct of 10,000 mg.min.m^{-3} (○).

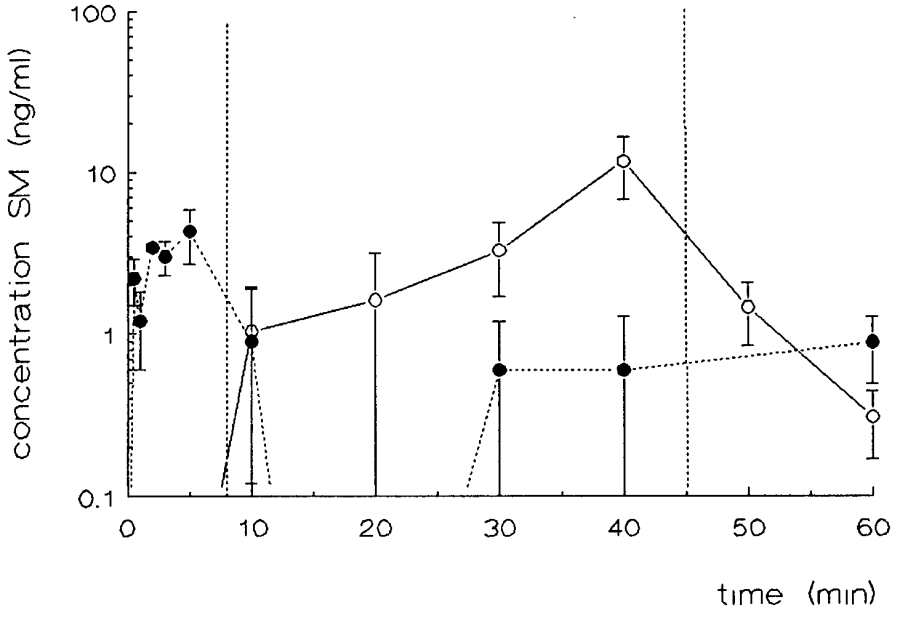

Figure 52a. The first 60 min of Figure 52 on an expanded time scale.

with 0.3 LD50. Especially in the skin very high concentrations were measured, as was expected. Furthermore, concentrations of SM of several ng/g were found in fat tissue at 10 min after ending the 45-min exposure, which is lower than in tissues that are more richly perfused with blood such as lung, liver and spleen. Due to insufficient data it cannot be established whether the concetration of SM builds up with time in fat tissue. The concentrations of 7-SM-gua in the tissues are lower than was anticipated on the basis of the concetrations of intact SM in these tissues. Prsently, we have no explanation for this discrepancy.

Protection by scavengers
Pretreatment with NAC or CIPE at doses of 5 mmoles per kg body weight increases the 96-h LCt50 of SM for 5-min nose-only exposure from 800 mg.min.m^{-3} to 1285 and 1125 mg.min.m^{-3}, respectively. This seems to suggest protective ratios of 1.6 and 1.4, but statistical evaluation with the Student's t-test showed that the LCt50 values for pretreated animals was not significantly higher than the value determined for unprotected animals. Lailey *et al.* (1991) showed that pretreatment of rats with NAC or CIPE protected animals against a lethal dose of PFIB. CIPE was considered as a promising compound for protection against inhaled reactive toxicants since it is distributed preferentially into the lung, unlike NAC. Recently, Langford *et al.* (1996) showed in lung slices that preincubation with CIPE does not protect cellular glutathione against SM. Sofar, the use of NAC and cysteine esters does not seem very promising for protection against SM. In view of the abovementioned findings with respect to the inhalation toxicokinetics of SM it is questionable whether this approach to pretreatment against the toxic effects of SM is the most appropriate one. However, it would seem worthwhile to test the efficacies of these scavengers against a percutaneous challenge with SM.

Efficacy of Topical Skin Protectants
From the results we obtained with USAMRICD Topical Skin Protectants 1511 and 2701 it can be concluded that both TSP's protect the skin of the hairless guinea pig reasonably well against the effects of SM vapor or liquid. The protection against SM vapor appears to be somewhat better than against a liquid challenge. Whereas the TSP's are equally effective against SM vapor, TSP 2701 provides more protection against liquid SM than TSP 1511. According to the technician who tested these TSP's on the hairless guinea pigs, the latter TSP was more difficult to apply onto the skin than the former, which may explain the difference in performance.
The results obtained for these two TSP's underscore the potential of the use of TSP's as a 'pretreatment' against the effects of SM exposure.
In view of the appearance of SM in blood during and after percutaneous exposure of hairless guinea pigs to SM vapor, one may wonder whether the experimental design for testing the TSP's was adequate. Since only 1-cm^2 areas were covered with TSP, it cannot be excluded that a fraction of the DNA adducts in the skin is formed by SM distributed to the skin via the blood. However, it has to be realized that the Ct to which the animals were exposed was nearly 7-fold lower in these experiments than in the percutaneous toxicokinetic studies. Since the concentrations of SM in blood of such a lower exposure are anticipated to be very low, it seems unlikely that the systemic route contributes significantly to the high adduct levels measured in the skin.

The toxicokinetic studies of SM that were performed within the context of this Cooperative Agreement provide useful information on how the compound behaves in the hairless guinea pig. The results obtained for SM in blood and for SM and 7-SM-gua in tissues generally do not disagree with each other. These data can be used as a basis for constructing a physiologically-based model for the toxicokinetics of SM in the hairless guinea pig. In order to be able to construct such a model, additional toxicant-specific parameters have to be measured, such as the tissue-blood partition coefficients for relevant tissues, numbers of binding sites, as well as rate constants of binding and hydrolysis. If such a model has been validated for the hairless guinea

pig,it can be scaled to other species, including man, and can be used to design strategies to improve pretreatment and therapy of intoxications with this chemical warfare agent.

V. CONCLUSIONS

1. SM can be extracted from blood by liquid-liquid extraction with ethyl acetate, by mixing on a whirlmixer followed by ultrasonication. The absolute recovery of SM is *ca.* 90 %, whereas the recovery relative to the internal standard D_8-SM is almost 100 %. The extraction is performed at 4 °C. The pH does not influence the recovery of SM in the pH 5-7.5 range. The concentration of chloride ions (1.7-34 M) does not significantly influence the recovery either.

2. Mass spectrometric (MS) detection via positive electron impact ionization under semi-single ion monitoring is the most sensitive detection principle for SM. Absolute detection limits of *ca.* 100 fg can be attained. Furthermore, MS detection is highly selective.

3. Our newly developed gas chromatographic (GC) configuration, based on automated on-column large volume injection, two-dimensional chromatography, and electron capture detection increases the daily sample throughput, whereas the large volume injection and sensitive detection mode allow for minimum detectable concentrations of SM in blood samples of *ca.* 25 pg/ml. In this configuration SM and D_8-SM are completely resolved. This complex and delicate configuration needs further optimization before it can be used in routine bioanalysis.

4. SM can be analyzed in blood samples by GC-MS. By using an on-column autosampler, the daily sample throughput is increased. The detection limit of 33 pg/ml blood is adequate to study the toxicokinetics of SM after i.v. administration of a dose corresponding with 1 LD50. However, the system is contaminated very rapidly in routine bioanalysis, resulting in deterioration of chromatographic performance and loss of sensitivity. Therefore, additional clean-up of the extracts is necessary.

5. Leading the ethyl acetate extract from blood over a SepPak Florisil® cartridge reduces the amount of contaminants, without loss of SM. However, this clean-up step is not sufficient to prevent serious contamination of the GC-MS configuration in routine bioanalysis.

6. Replacement of the on-column autosampler of the GC-MS configuration with a thermodesorption autosampler (TDAS) enables large volume injection and additional on-line sample clean-up by selecting appropriate desorption conditions. A detection limit for SM in blood below 5 pg/ml can be reached on a routine basis with this configuration.

7. Gas chromatography with pulsed-flame photometric detection (GC-PFPD) provides for a highly selective and sensitive method of analysis of SM in biological materials. When combined with thermal cold trap (TCT) injection, the detection limit (S/N=3) for SM in biological samples is 50 pg, which corresponds with 125 pg/ml for a 400-µl injection. Consequently, TCT-GC-PFPD is a valuable additional method for bioanalysis of SM, which was successfully applied to determination of the concentration of intact SM in tissue samples.

8. The immunoslotblot assay is a very selective and sensitive method of analysis for adducts of SM to guanine. At very high adduct levels, however, the method appears to be less reliable, since hardly any DNA can be isolated from such samples. For skin samples with very high adduct levels, immunofluorescence microscopy appeared to be a suitable alternative method to establish DNA damage.

9. The 96-h intravenous (i.v., vena jugularis) LD50 in hairless guinea pigs is 8.2 mg/kg (95 % confidence interval 7.1-8.8 mg/kg).

10. The lowest level of toxicological relevance was estimated from the lowest concentration of SM which forms DNA adducts in white blood cells to be *ca.* 1.6 ng/ml blood. This assumed lowest level of toxicological relevance of SM is well above the detection limit of the bioanalytical procedure.

11. The i.v. toxicokinetics of SM for a dose corresponding with 1 LD50 are characterized by a very rapid distribution phase and a very slow elimination phase, with half-lives of 0.9 and 347 min, respectively.

12. The concentration of SM in various tissues (lung, spleen, liver and bone marrow) exceeded that in blood shortly after i.v. administration of the agent at a dose corresponding with 1 LD50, indicating substantial partitioning of SM from the blood into the tissues.

13. The calculated toxicokinetic parameters for SM in the hairless guinea pig are in reasonable agreement with the scarce data reported in literature for the rat. However, preliminary data on the intravenous toxicokinetics of SM in the marmoset suggest a considerable interspecies variation.

14. Already at 3 min after i.v. administration of SM at a dose corresponding with 1 LD50, 7-SM-gua was present in blood, lung, spleen, bone marrow, liver and small intestine, as determined with an immunoslotblot assay. The highest initial adduct levels are observed in the lung, which are much higher than in blood. Most of the adducts disappear within 6 h after SM administration. However, at 48 h after administration significant adduct levels are still present in most tissues.

15. The i.v. toxicokinetics of a dose of SM corresponding with 0.3 LD50 showed the same general pattern as found for 1 LD50. Non-linearity of the toxicokinetics of SM in blood with the dose was observed. Concentrations of intact SM and 7-SM-gua in tissues were also considerably more than 3.3-fold lower than after i.v. administration of a dose corresponding with 1 LD50.

16. The apparatus for generation of soman and sarin vapor in air and for nose-only exposure of anesthetized, restrained guinea pigs was adapted to enable continuous generation of SM vapor concentrations up to 350 $mg.m^{-3}$ in order to perform nose-only exposure of anesthetized, restrained hairless guinea pigs.

17. The 96-h LCt50 of SM for 5-min nose-only exposure of anesthetized, restrained hairless guinea pigs appeared to be 800 $mg.min.m^{-3}$ (95-% confidence interval 700-920 $mg.min.m^{-3}$).

18. During and after 5-min nose-only exposure of anesthetized, restrained hairless guinea pigs to a dose of SM corresponding with 1 LCt50, the intact agent could not be detected in blood, whereas 7-SM-gua was barely detectable in blood and lung.

19. SM was measurable in blood during and after 8-min nose-only exposure of hairless guinea pigs to a Ct of 2,400 mg.min.m^{-3} (3 LCt50). Toxicokinetic evaluation of the concentration-time profile is tedious, and only possible by assuming two absorption processes, i.e., a very rapid one and a slow one. Concentrations of intact SM in tissues were very low. The highest concentrations were observed in lung and liver, shortly after ending the exposure. Measurable concentrations of 7-SM-gua were only observed in the blood and the lungs of the exposed animals.

20. Most DNA-adduct formation in the respiratory tract of hairless guinea pigs at 4 h after ending a 5-min nose-only exposure to 1 LCt50 SM had occurred in the larynx and trachea, whereas hardly any 7-SM-gua was found in the lung. This observation is in agreement with the observation that SM could not be detected in the blood of animals nose-only exposed to 1 LCt50 SM.

21. Histopathological evaluation of the respiratory tract of hairless guinea pigs at 4 h after ending a 5-min nose-only exposure to 1 LCt50 SM showed the damage to be mainly located in the upper airways, with hardly any damage in the lungs. These findings are in agreement with those for the distribution of the DNA adduct levels within the respiratory tract.

22. An apparatus was developed for percutaneous exposure of hairless guinea pigs to SM vapor in air, allowing the animal to breathe clean air, and with a possibility to draw blood samples from the carotid artery.

23. During and after a 45-min percutaneous exposure of anesthetized, restrained hairless guinea pigs to SM vapor, yielding a Ct of *ca.* 10,000 mg.min.m^{-3}, which according to literature corresponds with approximately 1 percutaneous LCt50, SM can be detected in the blood. The concentration-time profile in blood suggests the existence of a rapid and a slow absorption process. Shortly after ending the exposure, concentrations of intact SM exceeding that in blood were measured in the tissues. Rather low concentrations of 7-SM-gua were measured in most tissues. From most of the skin samples, DNA could not be isolated, indicating massive DNA damage which leads to cross-linking between DNA and proteins.

24. Pretreatment of hairless guinea pigs with 5 mmoles/kg of N-acetyl cysteine or cysteine isopropyl ester did not significantly increase the 96-h LCt50 of SM for 5-min nose-only exposure.

25. Topical Skin Protectants 1511 and 2701 protected hairless guinea pigs against skin damage caused by liquid SM and SM vapor. Based on the amount of DNA adducts in skin, the protection against a vapor challenge appeared to be almost complete, whereas protection against liquid SM was somewhat less effective. Whereas the TSP's performed equally well against SM vapor, TSP 2701 performed better against liquid SM than TSP 1511.

26. The adverse effects due to respiratory exposure to SM appear to be more of a local than of a systemic nature. In order to improve the protection against the respiratory effects of SM, the uptake and distribution of SM after nose-only exposure need to be studied further in various species.

27. A systemic intoxication with SM is more likely to occur from a percutaneous exposure than from a nose-only exposure, at least in the hairless guinea pig.

28. On the basis of the results of this study it seems worthwhile to test the efficacy of scavengers such as N-acetyl cysteine and cysteine isopropyl ester against the systemic effects resulting from percutaneous exposure rather than from respiratory exposure to SM.

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